Cyclooxygenase-2 and Prostaglandins in Human Endometrial Function

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Declaration

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

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Abstract

A role for cyclooxygenase enzymes and prostaglandins (such as prostaglandin E_2) has been observed in benign endometrial pathologies (endometriosis, excessive blood loss and dysmenorrhoea) and in adenocarcinoma. Cyclooxygenase and prostaglandin E synthase enzymes catalyse the conversion of arachidonic acid to prostaglandin E_2. Once synthesised, prostaglandin E_2 mediates its effects via four G protein coupled receptors namely EP1, EP2, EP3 and EP4. These receptors signal via alternate and sometimes opposing pathways. The initial aim of the research presented in this thesis was to investigate the temporal expression and signalling of the prostaglandin E_2 pathway in the normal human endometrium across the menstrual cycle. Prostaglandin E synthase and prostaglandin E_2 were localised to glandular epithelial and endothelial cells. Similarly, expression of the prostaglandin E_2 receptors, namely EP2 and EP4, was temporally up regulated and co-localised to the glandular and vascular compartments. This was associated with enhanced cAMP turnover in response to exogenous prostaglandin E_2. In order to investigate further the role of cyclooxygenase-2 and prostaglandin E_2 in glandular epithelial cells we generated a stably transfected endometrial epithelial cell line (Ishikawa) overexpressing cyclooxygenase-2 in either the sense or antisense directions. Using these cell lines we observed enhanced secretion of prostaglandins E_2 and $F_{2\alpha}$ into the culture media of the cyclooxygenase-2 sense cells compared with the cyclooxygenase-2 antisense and wild type cells in response to exogenous arachidonic acid. Co-incubation of the cells with NS398 (specific cyclooxygenase-2 inhibitor) abolished the increase in prostaglandin synthesis. Overexpression of

cyclooxygenase-2 was accompanied with significantly elevated EP2/EP3 receptor expression. No differences were detected for EP1, EP4 and FP receptors. These results indicate a possible autocrine/paracrine action of cyclooxygenase-2 enzyme products on expression of prostanoid receptors such as EP2/EP3. During the course of my PhD, numerous reports were published implicating cyclooxygenase-2 and its products in angiogenesis through the expression of angiogenic factors such as vascular endothelial growth factor, basic fibroblast growth factor and angiopoietins. To investigate the potential role of cyclooxygenase-2 in regulation of endometrial angiogenesis, cDNA array technology was employed to identify differentially expressed genes that may be involved in vascular function. Using this technique, a total of 81 genes were differentially regulated including cathepsin D. Cathepsin D mRNA and protein expression were elevated in the cyclooxygenase-2 antisense cells compared with the sense and wild type cells. Cathepsin D is known to proteolytically cleave plasminogen to the antiangiogenic factor angiostatin. Hence, we investigated the generation of angiostatin from plasminogen in conditioned media collected from cyclooxygenase-2 sense, cyclooxygenase-2 antisense and wild type cells. The cleavage of angiostatin from plasminogen was markedly enhanced in conditioned media from cyclooxygenase-2 antisense cells compared with cyclooxygenase-2 sense and wild type cells. Co-incubation of plasminogen with pepstatin A, a selective cathepsin D inhibitor, markedly reduced the cleavage of angiostatin from plasminogen thus further implicating cathepsin D in the differential angiostatin production by the cyclooxygenase-2 sense and antisense cell lines.

In conclusion data presented in this thesis outline the temporal regulation of prostaglandin E_2 receptor expression and signalling in the human endometrium.

Moreover, we report a novel role for cyclooxygenase-2 in promoting angiogenesis through suppression of production of antiangiogenic factors such as angiostatin. The elevated expression of cyclooxgenase-2 observed in numerous endometrial pathologies may therefore play a crucial role in regulation of angiogenesis through expression of pro-angiogenic genes and inhibition of production of anti-angiogenic factors.

Abbreviations

AA	Arachidonic acid
ANOVA	Analysis of variance
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
AS	Antisense
ATP	Adenosine Triphosphate
bp	Base pairs
BSA	Bovine serum albumin
COX	Cyclooxygenase
cAMP	Adenosine 3', 5'-cyclic monophosphate
cDNA	Complementary deoxyribonucleic acid
CRE	cAMP response element
CRTH2	Chemoattractant Receptor homologous molecule expressed on Th2 cells
dH_2O	Distilled water
DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked ImmunoSorbent assay
eNOS	endothelial Nitric Oxide Synthase
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FAP	Familial Adenomatous Polyposis
g	gravity
GPCR	G – protein Coupled Receptor
H_2O	Water
IBMX	3-isobutyl-1-methylxanthine
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin

IP_3	Inositol triphosphate
JNK	c-Jun amino-terminal kinase
kb	Kilobases
kDa	Kilodaltons
LPS	Lipopolysaccharide
MAPK	Mitogen-associated protein kinase
MCP-1	Monocyte Chemoatactic Peptide-1
MEK	Mitogen-associated protein kinase / Extracellular signal-regulated kinasekinase
hMLH1	Human Mut L Homolog 1
MMP	Matrix Metalloptroteinases
NFκB	nuclear factor-kappaB
NP40	"Nonidet" P40
NK	Natural Killer cells
nm	nonometer
NSAIDs	Non-steroidal anti-inflammatory drugs
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PGES	Prostaglandin E synthase
15-PGDH	Prostaglandin Dehydrogenase
hPGDS	haematopoietic-type Prostaglandin D_2 Synthase
PGI_2	Prostacyclin
PGT	Prostaglandin transporter
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMSF	phenylmethylsulphonylfluoride
PPAR	Peroxisome proliferator-activated receptors
PTEN	Phosphatase and Tensin Homolog
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
S	Sense
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean

Taq	Thermus aquaticus
TBS	Tris buffered saline
TBST	Tris-buffered saline with Tween® 20
TNF	Tumour necrosis factor
Tris	Trizma base
TXA_2	Thromboxane A_2
VEGF	Vascular endothelial growth factor
WT	Wild Type
X-Gal	5-bromo-4-chloro-indoyl β-D-galactopyranoside

Chapter 1

General Introduction

1.1 Introduction

Menorrhagia; excessive blood loss in menstruation, dysmenorrhoea; associated with painful menstruation and endometriosis; where uterine tissue is found in sites outside of the uterus, are common benign gynaecological conditions (1). The underlying mechanisms of common gynaecological pathologies are poorly understood. A role for cyclooxygenase-2 (COX-2) and its products, the prostaglandins, in the pathology of these conditions has been proposed by a number of studies (2-7). The focus of this thesis is on the role of COX-2 and its products the prostaglandins in endometrial function. This introduction will give a brief overview of the structure, histology and normal function of the endometrium, the roles of COX-2 and prostaglandins in physiological and pathological states including normal endometrial function and the association with endometrial pathologies.

1.2 Uterus / Endometrial Histology

The uterus is the inverted pear shaped organ situated in the pelvic cavity. Developmentally, the uterus is a muscular expansion of the Müllerian duct. There are three distinct structural regions of the uterus namely the uterine fundus, the uterine corpus and the uterine isthmus. The uterine fundus and corpus have a three layered wall. The wall consist of the perimetrium; a thin membrane, the myometrium; a layer of smooth muscle and the endometrium; a glandular lining. The endometrium is composed of the stratum basalis and the stratum functionalis (see figure 1.1). The superficial functionalis layer consists of a lining epithelium and uterine glands with a

stroma embedded with a vascular tree. The functionalis layer is shed monthly during menstruation, conversely, the basalis layer is not shed during menstruation; and, it gives rise to the new functionalis layer post menstruation. The primary roles of the endometrium are to provide an implantation window, regeneration in the absence of pregnancy and to offer protection from invading pathogens (8, 9).

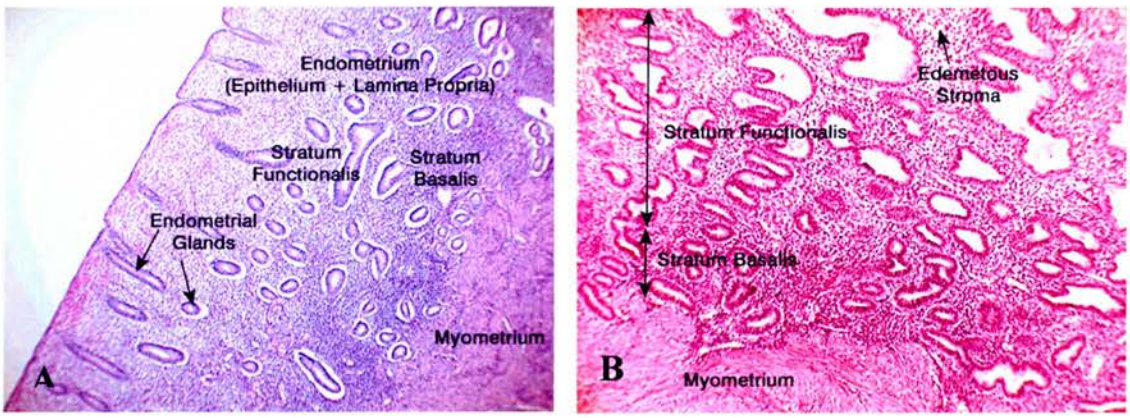

Figure 1.1 Histology of the human uterus **A** proliferative phase, **B** secretory phase. (Haemotoxylin- and Eosin stained, Reproduced from http://asb.aecom.yu.edu)

1.3 Steroidal Control and The Menstrual Cycle

In humans, the normal menstrual cycle lasts between 25 and 35 days (10). The menstrual cycle is ultimately under the control of the ovarian steroidal cycle. The cycle is divided into three phases: the proliferative or follicular phase, the secretory phase and in the absence of implantation, the menstrual phase; during which the functionalis layer of the endometrium is shed (see figure 1.2). The proliferative phase is post menstruation from day five of the cycle until ovulation which occurs on day 14, the predominant hormone is estradiol. The secretory or luteal phase occurs days 14-28 of a 28 day cycle and the predominant hormone is progesterone. In the absence of implantation, the fall in progesterone and estrogen as a result of the demise of the

corpus luteum initiate menstruation (11). The role of progesterone withdrawal in the induction of menstruation is highlighted by the observation that artificial maintenance of high progesterone levels results in the absence of menstruation. In contrast, administration of the progesterone receptor antagonist initiates uterine bleeding (12, 13). Interestingly, all mammals display withdrawal of progesterone and estrogen concurrent with corpus luteal regression. However, only humans, old-world primates and some bats menstruate (14).

1.3.1 Proliferative and Secretory Phases

Dating the endometrium according to histology and stage of the menstrual cycle was characterised by Noyes et al in the 1950s, the criteria are still used today (15). The initial repair of the endometrial surface occurs two days after the beginning of menstruation and is complete possibly within 48 hours (16). The early proliferative phase days 5-7 of the menstrual cycle, is typified with short narrow glands and a thin regenerating surface epithelium especially between the glands (15). An increased mitotic index is seen at day 5 of the menstrual cycle which remains high until the early secretory phase. (17). As proliferation continues, the endometrium thickens and reaches full thickness of 3 - 4mm by the time of ovulation. The mid proliferative glands begin to extend and curve with columnar surface epithelium. By late proliferative phase, the endometrium displays an undulant surface (15). After ovulation and early in the secretory phase, the lumina of the glands begin to fill with glycogen-rich secretions. The glands enlarge and become more tortuous. This is accompanied with a reduced mitotic activity in the epithelial cells (18, 19). The

stromal cells differentiate and decidualisation occurs. This process is marked by prolactin secretion (20). Decidualisation is crucial for the initiation and establishment of pregnancy (21). The process of decidualisation is thought to be partly mediated by progesterone and also by increased intracellular cAMP levels (21). In the peri implantation period, there is an influx of uterine specific natural killer cells (uNK) which are important in the regulation of the permissive invasion of cytotrophoblast cells (22).

1.3.2 Menstruation

In the absence of implantation and a decline in progesterone levels, menstruation is initiated. Menstruation is now thought to be a two staged process involving an inflammatory response and cells of the immune system (9, 11, 12). The initial phase of menstruation is progesterone dependent and probably reversible. Conversely, the second phase associated with the hypoxia and the activation of lytic systems is thought to be progesterone independent and irreversible (9). Withdrawal of progesterone is characterised by the upregulation of cytokines such as interleukin-8 (IL-8) and monocyte chemoatactic peptide-1 (MCP-1). Recently, gene array technology was employed to identify those chemokines important for leukocyte recruitment to the endometrium (22). The chemokine expression depended on the stage of the menstrual cycle. Chemokines that recruit marcrophage, NK and T cell population were upregulated in the periimplantation phase and early pregnancy. Upregulation of chemokines including IL-8 were observed in the pre menstrual phase and are thought to be responsible for recruiting leucocytes associated with

menstruation (22). Additionally, progesterone withdrawal results in enhanced COX-2 expression; and ultimately prostaglandin levels such as $PGF_{2\alpha}$, along with a reduced prostaglandin dehydrogenase (PGDH) expression (21, 23). The infiltrated leucocytes offer a further source of cytokines which in turn leads to the further recruitment of leucocytes (21). Within the uterus, matrix metalloptroteinases (MMPs) expression are upregulated post progesterone withdrawal. The MMPs are in part synthesised by stromal cells of the endometrium and also released by the infiltrated leucocytes (9). The increase in endometrial MMPs is concomitant with a significant elevation in gelatinase and collagenase activity at menstruation compared with other stages of the menstrual cycle. Moreover, inhibition of MMP activity in an endometrial explant in vitro model prevents its breakdown in a reversible manner (24). These observations support a role for the MMPs in menstruation and matrix degradation within the endometrium (25).

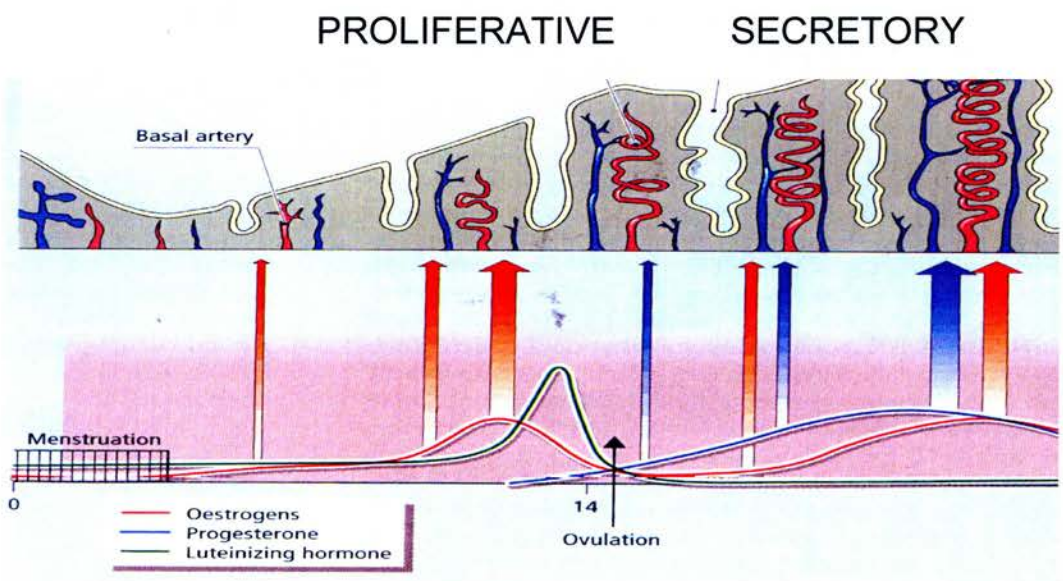

Figure 1.2 A Schematic diagram representing hormonal control and growth across the menstrual cycle in the human endometrium. (Adapted from Essential Reproduction (26)

1.4 Angiogensis in The Endometrium

Angiogenesis is the growth of new blood vessels from existing blood vessels. Physiological angiogenesis is most common in fetal growth and development. The endometrium is an unusual tissue in that it undergoes regular physiological angiogenesis which except for wound healing and in the corpus luteum is rare in normal adult tissues (27). The mechanism of angiogenesis in the endometrium appears to be different to that seen in other systems. Classically, angiogenesis has demonstrated a sprouting mechanism. Within the endometrium, the lack of staining for intergrin $\alpha_v\beta_3$; which is associated with sprouting angiogenesis, suggested an alternative mechanism. Intussucsceptive angiogenesis is now thought to be the main mechanism of angiogenesis within the endometrium with an elongation process in the functionalis layer (18, 27, 28). The process of intussucsceptive angiogenesis involves the lumen of a vessel dividing internally into two as endothelial cells proliferate and migrate inwards (27). Angiogenesis in the endometrium varies spatially and temporally across the menstrual cycle. During and post menstruation, repair occurs in the basalis layer, in the functionalis layer, spiral arterioles develop and coil during both the proliferative and secretory phases of the menstrual cycle. A capillary plexus forms just below the luminal epithelial surface and maximal blood flow is observed during the early and mid secretory phase. The capillary plexus forms in preparation for the implanting embryo (27). Endometrial angiogenesis is thought to partly be under the direct control of reproductive steroids and indirect control by angiogenic factors expressed by multiple cell types of the endometrium. Many growth factors have been identified in the endometrium (29). Potential

angiogenic regulators include vascular endothelial growth factor- A,(VEGF-A), VEGF-B, VEGF-C, angiopoietin-1 (Ang-1) and Ang-2. The contribution by individual growth factors and their cellular origin may be cycle dependent. It has been suggested that growth factors derived from endometrial NK cells may be important for angiogenesis during the secretory phase (30). Additionally, intra-vascular neutrophil derived VEGF-A may promote functionalis layer angiogenesis during the proliferative phase (29). Supernatants collected from cultured endometrium contain soluble factors such as VEGF-A that regulate angiogenesis. Moreover, those from the proliferative phase of the menstrual cycle promote a greater enhancement of pro angiogenic endothelial cell transcriptome change relative to the secretory phase (31). Abberant vascular function is thought to be involved in a number of endometrial pathologies such as menorrhagia as discussed in section 1.6.1. The expression of the angiopoietins-1 and 2 (Ang-1 and Ang-2) has been shown in the human endometrium. An altered ratio of Ang-1: Ang-2 has been demonstrated in menorrhagic endometrium with an overall shift to Ang-2 (32). Further research in endometrial angiogenesis may help to elucidate the underlying mechanisms of these pathologies (18).

1.5 Apoptosis in The Endometrium

Apoptosis is programmed cell death and is thought to be an important mechanism in maintaining homeostasis within the endometrium (33). Studies have detected apoptosis in the glandular epithelium in the late secretory and menstrual phases of the cycle, in contrast with the proliferative phase where little apoptosis has been

described (33). bcl-2 is a well studied apoptosis related protein and inhibits apoptosis; conversely, BAX a member of the bcl-2 family is pro apoptotic (33). The ratio of bcl-2 and BAX may play a role in controlling the susceptibility of a cell to apoptose (34). In the endometrium, it has been hypothesised that BAX and bcl-2 may be under the control of ovarian hormones with a negative correlation of apoptosis and estradiol in the proliferative phase (35). bcl-2 is expressed in endometrial glandular and stromal cells with maximal expression in the late proliferative phase. Conversely, BAX expression is low in the proliferative phase and rises in the secretory phase. Apoptosis may play a role in the pathophysiology of; the benign gynaecological condition, endometriosis (see section 1.6.1). Enhanced expression of anti apoptotic bcl-2 protein has been demonstrated in ectopic endometrial tissue compared with paired eutopic endometrium (36). An elevation in bcl-2 has also been reported in proliferative eutopic endometrium from women with endometriosis relative to endometrium from women with normal menstruation (37). It is still not known if alterations in apoptosis are important in the initiation of endometriosis or a secondary effect following the establishment of the disease (33). However altered apoptosis is likely to contribute to the pathology of endometriosis.

1.6 Endometrial Pathologies

1.6.1 Benign Endometrial Pathologies

Menorrhagia; dysmenorrhoea and endometriosis are common benign gynaecological conditions. Menorrhagia in the absence of identified uterine pathology is defined as

blood loss greater than 80ml lost per menstrual cycle. Up to 30% of women of reproductive age and 50% of perimenopausal women suffer from menorrhagia. In the developed world, menorrhagia is the most common cause of anaemia. Menorrhagia is the most frequent indicator for hysterectomy and accounts for at least 70% of 7000 hysterectomies performed annually in the UK (38-40).

Dysmenorrhoea associated with painful menstruation in the absence of pelvic pathology is very common effecting between 30% and 50% of menstruating women. In women with dysmenorrhoea, uterine hypercontractility has been observed resulting in reduced uterine blood flow and ischaemia. A correlation between minimal blood flow and maximal pain has been identified. (5)

Endometriosis is where endometrial glands and stroma are found in sites outside of the uterus including the pelvic peritoneum. This disease is of unknown aetiology and affects between 2 – 10% of women of reproductive age and 40% of women seeking infertility evaluation (41). Retrograde menstruation; which is the reflux of menses through the fallopian tube to the peritoneal cavity was first proposed by Sampson in 1927 and is the most widely accepted mechanism underlying endometriosis (41, 42). Whilst between 70-90% of menstruating women, display retrograde menstruation, the aberrant mechanisms resulting in endometriosis remain to be elucidated. It has been postulated that outflow obstruction of the menstrual flow may result in excessive retrograde menstruation leading to endometriosis. Moreover, both endometriosis and dysmenorrhoea may be manifestations of aberrant menstrual flow (41).

Of all gynaecologist referrals by GPs one third are due to menstrual disorders. In England and Wales, 25 000 procedures a year are performed for the treatment of menstrual problems. These benign gynaecological pathologies are a considerable health issue for women and place a financial burden on the health services (43).

1.6.2 Endometrial Carcinomas

Within the developed world, endometrial cancers are the most common female reproductive tract gynaecological malignancies and are associated with post menopausal women. Owing to symptoms such as abnormal vaginal bleeding, the disease is often caught early and this is reflected in the 86% five year survival rate. Approximately 80% of all endometrial cancers are endometrial carcinomas (44).

There are two recognised forms of endometrial carcinoma. Type I and Type II (45). Type I endometroid carcinomas are generally low-grade, low-stage and associated with an unopposed estrogen state. The tumours are of normal endometrial histology with the resemblance of proliferative endometrial glands (44). Type II serous carcinomas are poorly differentiated, associated within the setting of atrophy and are not linked with unopposed estrogen (44). These carcinomas are aggressive in nature and associated with a poor prognosis (46). The risk factors for type II endometrial carcinomas is advanced age and previous pelvic irradiation (47). The aetiology of endometrial cancer is poorly understood. For type I endometrial carcinomas, mutations primarily in exons 3,4,5,7 and 8 in the PTEN (Phosphatase and Tensin

Homolog) tumour suppressor gene are observed in up to 50% of the tumours. Interestingly, these mutations were not observed in either ovarian or cervical carcinomas suggesting the mutations are particularly significant in the pathogenesis of endometroid carcinomas (48). PTEN mutations have been demonstrated in 20% of hyperplastic lesions indicating these mutations may be an early event in the tumorigenesis of endometrial carcinoma (44). Microsatellite instability (MI) accompanied with hypermethylation of hMLH1 (Mut L Homolog 1) promoter; the DNA mismatch repair protein, is also observed in Type I endometrial carcinomas, especially in subjects with hereditary non polyposis colorectal cancer (44). Mutation of the K-ras oncogene has been idenfitifed in some endometrial carcinoma samples and is associated with positive expression of the progesterone receptor (49). Conversely, type II carcinomas rarely exhibit MI and PTEN mutations (48, 50). Mutations in p53 have been reported in endometroid carcinoma but are most commonly associated with type II serous carcinomas (46). Up to 90% of uterine serous carcinomas have been shown to display mutations in the p53 gene with a high percentage of p53 mutations also observed in intraepithelial carcinomas a putative precursor to uterine serous carcinomas (44, 46). This observation has led to the suggestion that p53 mutations are an early event in the pathogenesis of uterine serous carcinomas and may in part contribute to the aggressiveness of this disease (46).

1.6.2.2 Endometrial Cell Lines

A number of established cell lines of endometrial origin are available for in-vitro studies. These include HES and Ishikawa cells; both of which are used for studies described in later chapters of this thesis, and MFE-296 cells. The Ishikawa and MFE-

296 cell lines are derived respectively from well and moderately well differentiated human endometrial carcinomas (51, 52). Conversely, HES cells transformed spontaneously from a benign proliferative endometrial specimen (53). Positive cytokeratin expression by the cell lines indicates an epithelial origin (52, 53). Ishikawa cells have been demonstrated to express estrogen and progesterone receptors and grow in a monolayer on plastic. Culture of Ishikawa cells in the presence of physiological concentrations of estradiol is associated with enhanced proliferation, increased specific progesterone binding and increased alkaline phosphatase activity (52). Interestingly, MFE-296 cells express androgen receptors with low progesterone receptor expression (51).

1.7 Cyclooxygenase Enzymes and Prostanoids in Endometrial Pathology

The aetiology and underlying molecular mechanisms of the endometrial pathologies discussed above in sections 1.6 have yet to be fully established. A role for the cyclooxygenase enzymes and their products the prostanoids has been identified in both benign and neoplastic endometrial pathologies (40).

Women with menorrhagia display elevated synthesis of PGE_2 and expression of PGE_2 binding sites in the uterus compared with those with normal menstruation (2-4, 54). Moreover, there is an altered ratio in the profile of prostaglandins synthesised. PGE_2 relative to $PGF_{2\alpha}$ and PGI_2 relative to thromboxane A_2 synthesis are increased. A positive correlation with PGE_2 and menstrual blood loss has been observed. Nitric

Oxide is also elevated in menstrual flow from women with menorrhagia (54). It is suggested that an increase in vasodilatory agents may be contributing to the degree or duration of menstrual blood loss in women with menorrhagia (40). The profile of prostaglandins synthesised in women with primary dysmenorrhoea is altered with an elevation in both PGE_2 and $PGF_{2\alpha}$ in the menstrual blood flow (55). Endometrial explant studies have shown an increased capacity to synthesise $PGF_{2\alpha}$ in response to AA in explants from women with dysmenorrhoea compared with explants from women with pain free menstruation (56). This observation has suggested either an elevation in COX enzyme expression or activity or increased prostaglandin synthase expression (40). Furthermore, PGE_2 and $PGF_{2\alpha}$ have both been implicated in hyperalgesia in inflammatory models of nociception (57, 58). A role for COX and the prostaglandins in endometriosis has been suggested. Immunohistochemical studies have demonstrated an upregulation of COX-2 in endometriotic endometrium. An elevation in prostaglandins in the peritoneal fluid of infertile women with endometriosis has also been described (6, 7).

Numerous studies have implicated COX enzymes in tumorigenesis and the development of neoplastic pathologies (see section 1.13). COX-2 expression, is elevated in endometrial, cervical and ovarian carcinomas (59-63). Although, there have been conflicting reports on the upregulation of COX-2 in ovarian cancers (1). Elevated COX-1 expression has been reported in ovarian and cervical cancers but not endometrial (61, 64, 65).

These studies have led to the suggestion that inhibition of COX or the signalling pathway of prostanoids may be therapeutically useful in the treatment of endometrial carcinomas as suggested for other carcinomas such as colon (66, 67). The association of COX and prostaglandins with benign endometrial pathologies has led to the administration of inhibitors of COX such as mefanamic acid and ibruprofen as a first line treatment (39) The use of inhibitors of COX for menorrhagia and dysmenorrhoea results in both an analgesic effect and a reduction of 25% in menstrual blood loss (68). Inhibition of COX enzymes is discussed more fully in section 1.11.

1.8 Arachidonic Acid Metabolism

Prostanoid biosynthesis is dependent on the release of, the C-20 unsaturated fatty acid, arachidonic acid (AA) from the membrane phospholipids by phospholipase A_2 (PLA_2). The dietry precursor of AA is linoleic acid and AA is stored esterified to the membrane phospholipids (see figure 1.3).

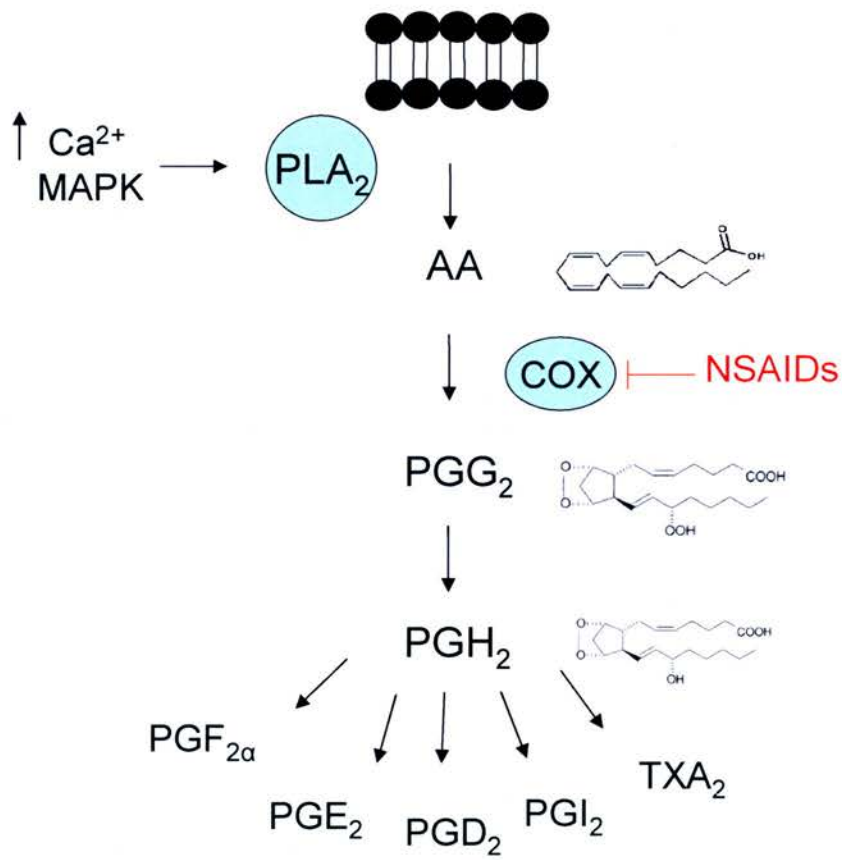

Figure 1.3. Schematic diagram of the prostanoid biosynthetic pathway

Numerous isoforms of PLA_2 have been identified; these include secretory PLA_2, cytosolic PLA_2 and Ca^{2+} independent PLA_2. Of those, the cytosolic PLA_2 ($cPLA_2$) is considered the key isoform in eicosanoid formation (69, 70). It has been suggested that $cPLA_2$ may play an important role in pathological states (70). $cPLA_2$ has recently been demonstrated to be overexpressed in human colorectal adenocarcinoma stromal cells, upregulate COX-2 mRNA expression and may play an important role in COX-2 mediated angiogenesis (71, 72). The translocation of $cPLA_2$ to the perinuclear membranes occurs following an elevation in cytoplasmic calcium levels and the activation of the MAPK intracellular signalling pathway (70). PLA_2 then catalyses the hydrolysis of the esterified form of AA and this cleaves AA from the cell membrane.

AA is metabolised by one of three pathways. Metabolism of AA by Cytochrome P450, COX enzymes and lipoxygenase (LOX) enzymes ultimately gives rise to prostaglandin epoxides, prostanoids and leukotrienes respectively. Collectively, prostaglandin epoxides, prostanoids and leukotrienes are known as eicosanoids (70). This thesis focuses on COX and prostaglandin mediated effects.

1.9 Cyclooxygenase Enzymes (COX)

The COX enzymes metabolise AA in a two stage process (see figure 1.3). Initially the cyclooxygenase activity oxygenates free AA to the unstable prostaglandin intermediate; PGG_2. The hydroperoxidase action of the COX enzymes then converts PGG_2 to the prostanoid precursor PGH_2 (73). Differences in the metabolism of AA

by the COX isoforms have been observed with COX-2 requiring less AA than COX-1 to be functional (74). NSAIDs including aspirin act to inhibit COX activity and subsequent prostaglandin release.

1.9.1 COX Structure

There are three isoforms of the COX enzymes namely, COX-1, COX-2 and COX-3 (70). COX-1 and COX-2 are derived from distinct genes. COX-1 is a 68 kDa protein and derived from a gene located on chromosome 9. The COX-1 gene is 25 kb in size with 11 exons and is transcribed to a 2.8 kb mRNA (70). The gene encoding COX-2 spans 8 kb and is located on chromosome 1 with 10 exons. The transcribed mRNA is 4.1 – 4.5 kb and gives rise to a protein of 72 kDa (70). The COX enzymes have been characterised in a number of species. Between species, COX-1 and COX-2 display around 80% - 90% homology. Furthermore, COX-1 and COX-2 from the same species share 60% - 65% sequence homology based on the amino acid composition (67). The COX-3 isoform has been most recently cloned. COX-3 mRNA is derived from the COX-1 gene and retains intron 1. In humans COX-3 is expressed as a 5.2 kb transcript (75).

COX enzymes are glycosylated integral membrane proteins (76). Post translational processing gives rise to either homodimers or heterodimers of COX-2 of 72 kDa and 72 kDa / 74 kDa respectively. However, post translational processing of COX-1 gives rise to a homodimer of 72 kDa. COX enzymes exist as dimers both structurally and functionally with superimposable crystal structures (77). Dimerisation is required for the catalytic activity and glycosylation for the cyclooxygenase and

peroxidase activities. Alternative sites of glycosylation of COX-2 gives rise to the 72 kDa and 74 kDa subunits. There are three structural domains namely, an N-terminal epidermal growth factor (EGF) like domain, a membrane binding domain and a globular catalytic domain with the heme-binding site (70). The COX enzymes bind one mole of high-spin ferric heme per mole monomer (76). One of the major structural differences between COX-1 and COX-2 is that COX-2 has a truncated signal peptide and an insertion of 18 amino acids at the C terminal. Although the functional significance of the insertion of 18 amino acids at the C terminal is unknown, it has been suggested it may be important for targeting COX-2 to the nuclear membrane (76, 78).

1.9.2 COX Biosynthetic Signalling Pathways

Mice knockout studies where either the COX-1 or COX-2 genes have been disrupted and pharmacological inhibition of COX enzymes demonstrate divergent effects (79-81). These observations suggest the individual COX enzymes may have distinct physiological roles. The COX enzymes share similar catalytic mechanisms and kinetics. However, the COX-2 active site is approximately 20% bigger than the COX-1 site (82). The difference in the size of the active site has been exploited in the generation of COX-2 selective inhibitors. COX-1 exhibits negative allosterism at low AA concentrations. Prostaglandin synthesis can be stimulated with 1μM AA in cells expressing COX-2, however, COX-1 generated prostaglandins require AA in concentrations of at least 10μM (83). It has been suggested that this may allow COX-2 to compete more effectively for AA in systems where both enzymes are expressed.

The COX enzymes are found in the endoplasmic reticulum, and the nuclear membrane (84). There is some evidence that COX-2 is more associated with the nucleur membrane compared with COX-1 (84). This has given rise to the suggestion that COX-2 products may be involved in gene transcription or regulation at the nuclear level possibly via nuclear peroxisome proliferator-activated receptors (PPARs) (84, 85). Thus, indicating an intracrine role for PGs arising from COX-2 (84). In addition, COX-1 and COX-2 products also signal via GPCRs on the cell surface. There is evidence that COX-1 and COX-2 preferentially metabolise AA to specific prostaglandins with COX-2 shifting the balance to the production of PGE_2 and PGI_2 (86). This observation may in part due to the localised expression of the prostaglandin synthases. PGI synthase and mPGE synthase both associate with the perinuclear membrane where COX-2 is more concentrated (70, 74). The cellular location of synthases can depend on the mode of stimulation. In HEK293 cells, stimulation with A23187 results in haematopoietic-type Prostaglandin D_2 Synthase (HPGD) synthase translocating to the endoplasmic reticulum and preferentially coupling with COX-1. Conversely, stimulation with IL-1β, HPGD synthase is translocated to the perinuclear envelope and results in preferential coupling with COX-2 (74). Moreover, the differences in the metabolism of AA and the cellular locations of COX-1, COX-2 and the terminal prostaglandin synthases may contribute to divergence of the apparently similar biosynthetic systems for COX-1 and COX-2 (70) .

1.10 Regulation of COX Enzymes

1.10.1 COX-1

COX-1 was originally purified in 1976, later being cloned in 1988. COX-1 is constitutively expressed in many cell types and is classically associated with physiological and housekeeping functions. Constitutive expression of COX-1 in endothelium, monocytes, platelets, renal collecting tubes and seminal vesicles has been demonstrated at high levels (87). An elevation in the expression of COX-1 in differentiating cells has been observed suggesting COX-1 is developmentally regulated (88). Interestingly, recent studies have shown COX-1 to be inducible in certain cancers (64, 65, 89-92). The COX-1 promoter does not contain a TATA or CAAT box. The possible transcriptional regulatory elements identified in the COX-1 promoter include Sp1, AP-2, NF-IL-6 and GATA (70, 87). Two Sp1 cis-regulatory elements in the COX-1 promoter have been recognised and are thought to contribute to constitutive expression of COX-1. The Sp1 sites bind the trans-activating Sp1 protein. Deletion of one of the Sp1 sites results in a 50% reduction in transcription increasing to 75% reduction where both Sp1 cis-regulatory elements are deleted (93).

1.10.2 COX-2

Unlike COX-1, COX-2 is an immediate gene and is rapidly induced by growth factors, cytokines and phorbal esters and is generally associated with pathological conditions (73, 79, 94, 95). The stimulus to induce COX-2 is dependent upon cell

type and related to the relevant physiological processes. For instance in the kidney, COX-2 expression is upregulated in response to increase salt concentration, however, in granulosa cells, FSH and LH induce COX-2 expresison (96-98). Under physiological conditions, COX-2 is normally expressed at almost undetectable levels, however, COX-2 is constitutively expressed in the brain, kidney, ovary and uterus (99-102). Further evidence for a role of COX-2 in homeostasis comes from studies in COX-2 knockout mice which display systemic abnormalities as discussed in section 1.12 (103). The COX-2 gene promoter region contains a number of identified transcription elements namely, NF-IL-6, AP-2, Sp1, two NF-$\kappa\beta$, CRE and E-box (70).

The MAPK and NF-$\kappa\beta$ signalling pathways are important in the control of COX-2 expression. The MAPK signalling cascade is divided into three pathways: ERK1/2, JNK/SAPK and p38 MAPK. Activators of the MAPK signalling pathways include growth factors, inflammatory mediators such as lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNFα) (77). Inhibition of ERK 1/2 MAPK in monocytes stimulated with LPS inhibits COX-2 expression. LPS stimulated monocytes treated with the p38 MAPK inhibitor results in the destabilisation of COX-2 (77). These studies indicate that in LPS stimulated monocytes, COX-2 expression and post translational regulation is dependent on the ERK 1/2 and p38 MAPK pathways. The JNK/SAPK and p38 signalling pathways have been demonstrated in different cellular models to mediate the upregulation in COX-2 expression by IL-1. In HeLa cells (cervical adenocarcinoma cells) inhibition of the p38 pathway attenuated COX-2 induction by IL-1 (95). Interestingly, the prostaglandin PGE_2 has been demonstrated

to stabilise COX-2 mRNA resulting in elevated COX-2 protein expression via the p38 MAPK pathway (104). Stabilisation of COX-2 mRNA by p38 MAPK is thought to be as a result of inhibition of deadenylation. Moreover, inhibition of p38 MAPK rapidly destabilises COX-2 mRNA.(104-106).

COX-2 induction by a variety of agents and conditions including IL-1β in osteoblastic cells and hypoxia has been shown to be mediated via the NF-$\kappa\beta$ pathway (77). Glucocorticoids inhibit COX-2 induction via AP-1 and NF-$\kappa\beta$ dependent transcription. Within the human endometrium, it is thought the negative regulation of COX-2 expression by progesterone is partly mediated via the NF-$\kappa\beta$ pathway (107, 108). Further evidence for the role of the NF-$\kappa\beta$ pathway in COX-2 expression is demonstrated by mutations in the NF-$\kappa\beta$ cis-regulatory region resulting in reduced COX-2 induction by TNFα stimulation (77).

1.10.3 COX-3

COX-3 displays a different pharmacological response compared with COX-1 and COX-2 and appears to be most sensitive to drugs with low anti inflammatory properties (75). The role and regulation of COX-3 in human physiology and pathological conditions has still to be elucidated.

1.11 Pharmacological Inhibiton of COX Enzymes

The NSAIDs block the synthesis of COX derived prostaglandins by inhibiting COX activity. There are essentially two groups of NSAIDS, non selective that block both COX-1 and COX-2 activity such as aspirin and ibuprofen and the COX-2 selective inhibitors which include celecoxib and NS398 (77). Whilst the non selective NSAIDs block the activities of both COX-1 and COX-2, there is evidence that they bind more tightly to COX-1 than COX-2 (109). The NSAIDs compete with AA to bind to the COX active site. NSAIDs block COX activity by one of three mechanisms. Ibuprofen like drugs act by rapid, reversible binding; time dependent, high affinity, slowly reversible binding is shown by drugs such as flurbiprofen or acetylation of Ser530 of which aspirin is the only drug that acts in this way (69, 77). Acetylation of Ser530 irreversibly prevents AA from binding to the substrate binding site thus requiring new protein synthesis. Other COX inhibitors demonstrate competitive binding and upon disassociation, COX levels return to basal (77). At higher concentrations, NSAIDs mediate their effects via alternative actions such as inhibition of IκB kinase-β preventing the activation of genes by NFκ β (110)

NSAIDs are used as anti-inflammatory, antipyretic and analgesic agents (111). Mefenamic acid an NSAID is the first line treatment for common gynaecological conditions such as menhorragia (39). Furthermore, use of NSAIDs has been shown to reduce the risk of colorectal cancer and reduce the size and number of colorectal polyps in patients with FAP. The use of NSAIDs has been linked with a 40-50% reduction in deaths from colorectal cancer (112). This has led to the suggestion that

NSAIDs are therapeutically useful in the treatment of carcinomas in a variety of tissues including the endometrium (66, 113).

Non selective NSAIDs have been associated with side effects including ulceration of the gastrointestinal tract and renal failure in patients with severely compromised renal function (114). The adverse side effects associated with non selective COX inhibition are thought to be due to the inhibition of the normal physiological actions of COX-1. These observations prompted the development of COX-2 selective inhibitors. COX-2 selective inhibitors are generally better tolerated than the non selective NSAIDs and display inhibition of the production of prostaglandins associated with COX-2 activity (115, 116). As previously mentioned the active binding site in COX-2 is approximately 20% bigger than in COX-1 and consists of an extra side pocket. It is this feature that allows COX-2 to accept a wider range of substrates than COX-1 and is exploited by COX-2 inhibitors. This is confirmed by restricting the size of the binding site, by substituting the Val523 to an isoleucine, COX-2 no longer shows affinity for the COX-2 selective inhibitors (18).

Recently concerns were raised about the safety of COX-2 selective inhibitors. Most noteworthy was the withdrawal of rofecoxib owing to its link with increased risk of myocardial infarction and strokes (117). The safety of other COX-2 selective NSAIDs has also been questioned. There is evidence that this class of drugs is heterogeneous in its actions (118). Rofecoxib is highly targeted to COX-2 and also displays one of the longest half lives (117). Celecoxib has been shown to inhibit proliferation and apoptosis in human vascular endothelial cells, whilst, rofecoxib

displayed neither of these effects (118, 119). Whilst more studies will be needed to confirm the safety of other COX-2 inhibitors, the differences shown in the actions mediated between the COX-2 selective inhibitors suggest they may still offer a useful therapy (117).

1.12 COX Knockout Studies and Effects on Reproduction

Further insights into the roles of the COX enzymes have come from COX knockout studies. COX-1 as mentioned in 1.10.1 is considered a housekeeping gene and associated with maintaining homeostasis. Despite this, disruption of the COX-1 gene in mice is coupled with surprisingly few deleterious effects (120). COX-1 is considered important in maintaining gastric integrity, kidney function and platelet aggregation. Surprisingly, COX-1 (-/-) mice displayed no gastric or intestinal ulceration or renal dysfunction. Moreover, treatment of the COX-1 (-/-) mice with the non selective COX inhibitor indomethacin was associated with reduced gastric ulceration compared with wild type mice (120). COX-1 (-/-) mice do display an altered inflammatory response to AA and reduced platelet aggregation. Furthermore, in mice, COX-1 and prostaglandin activity is crucial for parturition and pup survival. COX-1 (-/-) demonstrate delayed parturition and neonatal death (121). Whilst not demonstrated by Langenbach et al (122), other studies involving COX disruption report a compensatory action by the remaining COX activity (120, 121, 123). COX-2 as outlined in 1.10.2 is generally considered to be involved in pathological conditions with limited housekeeping functions. However, in contrast with COX-1, disruption of the COX-2 gene is not coupled with an impaired inflammatory response but is

linked with severe renal abnormalities and multiple reproductive failures (80, 103). Whilst male COX-2 (-/-) mice showed normal fertility, ovulation, fertilisation, blastocycst implantation and decidulisation are all compromised in female COX-2 (-/-) mice (80, 103). PGE_2 receptors expressed in the COX-2 null mouse uterus are comparative with wild type. Moreover, infused PGE_2 failed to induce decidulisation, thus suggesting other COX-2 products may be important in successful decidulisation. As previously mentioned, COX-2 is more associated with the nuclear membrane than COX-1 (85). Failure of PGs to restore COX-2 functions in a cell model has prompted the suggestion that nuclear prostanoid receptors play an important role in conferring COX-2 actions (80). Pharmacological selective inhibition of COX-2 showed reduced ovulation, fertilisation and implantation although these effects were not as dramatic as those seen in the COX-2 knockout mice. Simultaneous inhibition of COX-1 and COX-2 with NSAID treatment augmented the negative effect on implantation and decidulisation suggesting a compensatory action of COX-1 (81). In humans, there is some evidence that use of COX-2 selective inhibitors can impair fertility, cessation of the use of these drugs restores fertility (124). These studies demonstrate that COX-2 plays an important physiological role in renal and reproductive systems. The COX knockout mouse models highlight the oversimplification in categorising COX-1 exclusively as a housekeeping gene and COX-2 solely being involved in pathological states.

1.13 COX Enzymes in Cancer and Relevance of 15-PGDH

Over recent years, there has been mounting evidence for a role of the COX enzymes in carcinogenesis and the progression of the disease. Initial circumstantial evidence of a role for COX enzymes in cancers came from experiments in the 1980s. Prostaglandin synthesis was demonstrated to be elevated in human breast carcinomas and correlated with neoplastic cell density and tumour invasion (125). Epidemiological studies reported a 40-50% reduction in the risk of contracting colorectal disease in subjects with long term aspirin use (126). Further studies have shown a similar negative correlation between NSAID use and reduced death rates from cancers of the esophagus, stomach, breast, lung, prostate, urinary bladder and ovary (78). Familial Adenomatous Polyposis (FAP), an autosomal dominant disorder is characterized by the formation of hundreds of colorectal adenomas that progress to colorectal cancer. Patients with FAP treated with NSAIDs show a marked reduction in the number of colorectal polyps (127-129). Moreover numerous studies have now demonstrated an upregulation of COX-2 expression in carcinomas including colorectal, head and neck, oesophagus, pancreas, lung, prostate, bladder, and female reproductive cancers; endometrial, cervical and ovarian (61, 92, 130-133). An in-vivo model has demonstrated the growth of tumours in COX-2 null mice injected with lewis lung carcinoma cells is attenuated when compared with wild type cells (134). Furthermore, a significant reduction in VEGF production was also reported in the COX-2 null mouse fibroblasts. These effects in the COX-2 null mice were inducible in wild type mice treated with a COX-2 selective inhibitor (134). Interestingly, overexpression of COX-2 in a mouse skin tumour model resulted in a

suppression of tumour development. This observation suggests an alternative involvement for COX-2 in skin tumorgenesis (135).

It is now well accepted that delivery of nutrients and consequently tumour growth is dependant on angiogenesis and ultimately a vascular supply to the tumour (136). Putative mechanisms by which COX-2 influences tumour growth came from experiments in rat intestinal cells stably transfected with the COX-2 cDNA. Overexpression of COX-2 was associated with an elevation in the synthesis of prostaglandins. The increased generation of prostaglandins was accompanied with a decrease in apoptosis, enhanced cellular proliferation and an upregulated expression of angiogenic factors (137-139). Once synthesised, the angiogenic factors act in a paracrine manner to promote endothelial cell migration and microvascular tube formation (138). Moreover, treatment with NSAIDs in the overexpressing COX-2 models resulted in restoration of the basal apoptotic rate and an inhibition in angiogenic factor production thus supporting the notion that the protective effects of NSAIDs are mediated via their actions on the COX enzymes and reduced prostaglandin synthesis (137).

Until recently, the elevated expression of the COX products the prostaglandins in cancer models was thought to be due to the overexpression of COX-2 (140). However, catabolism of prostaglandins is dependent on the rate limiting enzyme, 15 – Hydroxyprostaglandin dehydrogenase (15-PGDH). The local accumulation of PGE_2 is most likely to depend on a balance between COX expression and 15-PGDH (140, 141). The expression of 15-PGDH has been shown to be markedly reduced in

colon cancer samples relative to normal colon giving rise to the suggestion that an upregulation of COX-2 in concert with a down regulation of 15-PGDH is required in colon cancer development (140, 142). The underlying molecular mechanisms have yet to be fully elucidated. However, TGF-β a known colon tumour suppressor induces 15-PGDH expression and this action may contribute to its tumour suppression action (141). Conversely, EGF has been demonstrated to down regulate 15-PGDH. A feed forward mechanism has been suggested for accumulating PGE_2 whereby COX-2 derived PGE_2 transactivates the EGFR. In turn this results in decreased 15-PGDH and an increased local PGE_2 concentration.

Whilst there is a wealth of evidence supporting a role for COX-2 in tumorigenesis, there is now evidence that COX-1 is also upregulated in certain carcinomas. Inhibition of COX-1 in a mouse FAP model is associated with a reduction in the number of intestinal polyps (79). Moreover, selective inhibition of COX-1 shows protective effects against colon cancer (78). These studies demonstrate that both COX isoforms play a role in tumorigenesis. Futher studies are required to establish the contribution of each isoform to tumour promotion (78).

1.14 Prostaglandins

Early experiments by Ulf von Euler led to the discovery of prostaglandins in the 1930s (73). Prostaglandins belong to the prostanoid group of the eicosanoids. Prostanoids comprise of the prostaglandins which contain a cyclopentane ring and thromboxanes with an oxane ring and are derived from C-20 fatty acids such as AA

by the cyclooxygenase enzymes. All the prostanoids contain a trans- double bond at C13-C14 and a hydroxyl group at C15 (143). Modifications of the cyclopentane ring gives rise to the different prostaglandin types e.g. PGE and PGF series (144). The prostaglandins include PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2. Once AA is metabolised as described above to PGH_2, PGH_2 acts as the substrate for prostanoid synthesis. PGH_2 is acted on by synthases specific to each prostanoid for instance PGE synthase (PGES) catalyses the conversion of PGH_2 to PGE_2. Most cells of the body synthesise prostaglandins (73). The specific prostaglandins synthesised depends on the prostaglandin synthases present in the cell or tissue with the profile of prostaglandin synthases present thought to be cell specific (77). Once synthesised, prostaglandins acting in an autocrine / paracrine manner are transported out of the cell by facilitated transport by the prostaglandin transporter (145, 146). Prostaglandins have a relatively short half life and act local to the site of synthesis (69).

Prostaglandins are important in a wide range of physiological and pathological processes (77). In a physiological setting, prostaglandins are involved in maintaining cardiac function, renal function, mediation of pain and reproduction. The prostaglandins have been implicated in the pathogenesis of cancer, rheumatoid arthritis, Creutzfeldt-Jakob Disease (CJD) and Alzheimer's disease (77, 147, 148). The roles of prostaglandins and the signalling pathways that mediate their effects are discussed in the following sections.

1.15 Prostaglandin Receptors

Prostanoids mediate their effects via G protein coupled receptors on the cell surface (149).The first PG receptor to be cloned was the TXA_2; TP receptor. In humans, a total of 9 prostanoid receptors encoded by separate genes have been described (143). Three groups of receptors have been described, the first IP, EP2, EP4 and DP are relaxant receptors and couple with G_s to stimulate cAMP turnover. The second group comprises of TP, EP1 and FP receptors and are considered the contractile receptors and couple with G_q to increase intracellular Ca^{2+} levels. Lastly EP3 and CRTH2 couples with G_i and inhibits adenylate cyclase and cAMP turnover (143, 149). These groups however, do contain exceptions where receptors can couple to multiple G-proteins (143). The prostaglandin receptors display only a 20% - 30% homology between them. Even when comparing EP1, EP2, EP3 and EP4, the homology is still very low. Studies have shown that the prostanoid receptors signalling via the same pathways demonstrate higher homology than when comparing those sharing the same ligands (144). With the exception of the recently discovered DP2 receptor, the receptors are G protein coupled with seven transmembrane domains and members of the rhodopsin-type receptor superfamily (149). The prostanoid GPCRs show selective ligand binding specificity. PGE_2, PGI_2, $PGF_{2\alpha}$, PGD_2 and TXA_2 act on the EP1-4, IP, FP, DP1 and TP receptors respectively (see figure 1.4). The DP2 receptor; or chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2), lacks homology with the prostaglandin receptors and is more closely related to the chemoattractant GPCRs than other prostaglandin receptors (148, 150, 151) The EP3 and more recently the FP receptors have been demonstrated to have alternative splice

variants (149, 152, 153). The receptors signal via multiple pathways. Co-expression of prostanoid receptors signalling via opposing pathways is often present. This has been suggested to act as means to maintain homeostatic control and in part explain the diverse and complex actions elicited by prostaglandins (154).

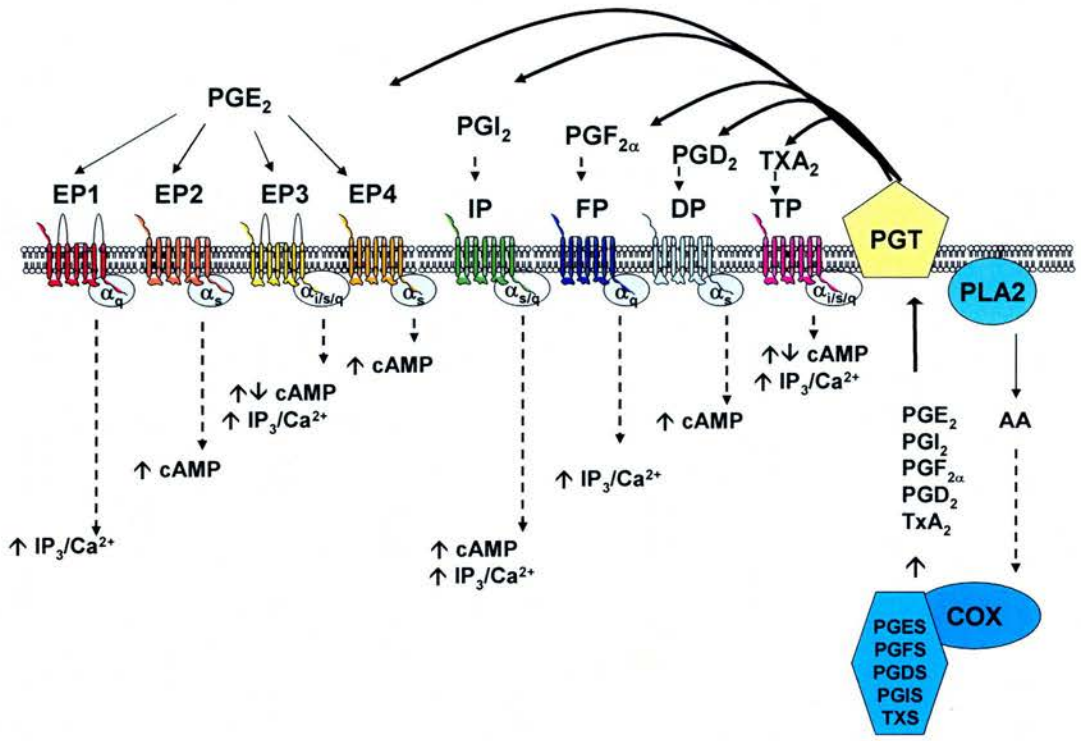

Figure 1.4 Schematic representation of the cyclooxygenase and prostanoid biosynthetic and signalling pathway. (Reproduced from Jabbour and Sales 2004 (155))

Recently the nuclear located peroxisome proliferator-activated receptors (PPARs) have been identified (156). PGJ_2 and PGI_2 are known ligands of these receptors (85, 156, 157).

1.15.1 EP Receptors

PGE_2 elicits its effects via the EP receptors. There are 4 recognised EP receptors termed EP1, EP2, EP3 and EP4 (149). EP3 displays at least eight alternative splice variants (158). These receptors act via different signalling and sometimes opposing pathways even within the same tissue (154, 159). PGE_2 mediated actions via EP

receptors expressed on the cell surface has been widely described. PGE_2 is known to have diverse physiological functions including an involvement in maintaining gastric integrity, preserving renal function and has a role in controlling blood pressure (149). The presence of functional EP receptors on the nuclear envelope has been identified (160, 161). Activation of these receptors may mediate alternative effects than receptors expressed on the cell membrane. Furthermore, this identifies a possible intracrine action of PGE_2 and would enable a PGE_2 to mediate direct regulation of gene transcription (162). Regulation of eNOS via nuclear located EP3 receptors has been shown in cerebral microvessel endothelial cells. This action is not thought to be cell surface receptor mediated (162).

1.15.1.1 EP1

EP1 receptor distribution in the mouse has been reported in the kidney, lung and stomach (163). The EP1 receptor elicited effects include contraction of smooth muscle; in tissues such as the respiratory tract and the myometrium, and neurotransmitter release (164). Pain elicited by PGE_2 is thought to be mediated in part via the EP1 receptor (165). EP1 receptors are coupled with G_q and activation results in increased intracellular Ca^{2+} levels (149). Interestingly an EP1 splice variant receptor with six transmembrane domains has been demonstrated in the rat (rEP1-variant) that binds PGE_2. Co-expression of the rEP1-variant with either rat EP1 receptor or EP4 receptor in CHO cells resulted in a reduced Ca^{2+} or cAMP accumulation respectively (166). It has been hypothesised that the rEP1-variant receptor may alter the efficiency of signal coupling of EP receptors and limit the

action of PGE_2 (166). A six transmembrane splice variant of the FP receptor has been described in the human (153).

1.15.1.2 EP2

The EP2 receptor is the least abundantly expressed of the EP receptors (149). In the mouse, EP2 receptor expression has been confirmed in various tissues including the uterus, spleen and lung. The EP2 receptor has been shown to be involved in ovulation, implantation and inflammation. The mediated effects by EP2 are thought to be inhibitory (164). Activation of the EP2 receptor results in increased cAMP generation via coupling to G_s (167). Recently, cross communication with the epidermal growth factor receptor (EGFR) and EP2 has been reported resulting in the activation of the MAPK pathway (168). The EP2 receptor can be upregulated as demonstrated in macrophages treated with LPS (169). Gonadotropins in uterine luminal epithelial cells have also been shown to induce EP2 mRNA expression (170). EP2 mRNA expression is upregulated in a mouse model for familial adenomatous polyposis (171)(FAP). Moreover, PGE_2 via the EP2 receptor was demonstrated to up regulate COX-2 and thereby promote a feed forward mechanism to further enhance PGE_2 synthesis (171). In contrast with EP4, following short term ligand binding, the EP2 receptor does not undergo desensitisation (172). This may indicate EP2 is important in mediating prolonged responses to PGE_2 (172).

1.15.1.3 EP3

EP3 receptor expression is highest in the kidney, uterus, adrenal gland and stomach tissues (149). Effects mediated via the EP3 receptor include smooth muscle contraction, modulation of neurotransmitter release, inhibition of gastric acid secretion and contributes to the maintanence of renal function (164). There are currently eight identified EP3 splice variants which arise from alternative C terminal splicing (149, 173). Activation of the EP3 receptor is associated either with a G_s mediated increase in cAMP or a G_i linked decrease in cAMP depending on the splice variant (149). The physiological significance of the EP3 splice variants remains to be established, however, there is evidence that the splice variants are expressed in a tissue dependant manner (174). Internalisation of EP3 receptor on ligand binding is dependant on the splice variant (173). This may indicate differences in the physiological functions between the splice variants depending on whether the PGE_2 is secreted over a short or prolonged period of time (173). The expression of multiple EP3 splice variants has been reported in the myometrium and the ratio of expression of the EP3 variants varies in gravid human myometrium (158, 175). This implicates EP3 receptor signalling in myometrial contractility (175). Furthermore, this suggests that a balance of expression for EP3 receptors is important. EP3 elicited effects have been implicated in a model of tumour angiogenesis and growth (176). Further studies would be required to determine if an altered ratio of EP3 splice variants is observed in pathological conditions.

1.15.1.4 EP4

Initially, two receptors were cloned and termed EP2 (149). Further studies demonstrated one receptor to be sensitive to butaprost and the other insensitive. The receptor cloned by Honda et al (177) and insensitive to butaprost was termed EP4. The butaprost sensitive receptor remained classed as the EP2 receptor (143, 177, 178). As with the EP2 receptor, activation of the EP4 receptor results in increased cAMP generation. EP4 receptors have a higher affinity for PGE_2 than EP2. However, in response to exogenous PGE_2, HEK cells overexpressing EP4 receptors demonstrate lower cAMP generation than HEK cells overexpressing EP2 receptors (179). EP4 mRNA expression is highest in the small intestine but is present in various other tissues including the lung, kidney uterus and brain (180). There is evidence from in vitro studies in rat colon epithelial cells and EP4 knockout mice linking EP4 receptors with maintenance of gastric integrity (181, 182). Vasodilatory effects and a role in closing the ductus arteriosus have been described for the EP4 receptor (149).

1.15.2 FP Receptors

$PGF_{2\alpha}$ elicits its effects via the FP receptor and is important in reproductive systems (148). FP receptor expression has been reported to be greatest in the corpus luteum (144). Recently, the temporal expression and signalling of FP receptors within the human endometrium has been described (183). A FP splice variant receptor has been identified in a number of species. FP splice variant expression has been described in

ovine corpus luteum; namely the FP_A and FP_B variants (184), bovine corpus luteum and most recently, a human FP splice variant (hFPs) has been identified (150, 153). Interestingly, Northern blot and immunohistochemical studies have demonstrated alternative ligand mediated internalisation of the FP_A and FP_B receptors (185) hFPs at mRNA and protein level in tissues such as the heart, skeletal muscle and the placenta (153). The newly reported hFPs and bovine FP splice variants appear to exist as six transmembrane receptors; with an extracellular carboxyl terminus, as opposed to the previously described seven transmembrane FP receptor. The function and physiological significance of the truncated hFPs receptor has yet to be elucidated (153). Activation of the FP receptor results in G_q coupled signaling, activation of phospholipase Cß (PLCß), release of inositol trisphosphate (IP3) and diacylglycerol. Signaling of the FP receptor resulting in the activation of Rho in a G_q independent manner has also been described (186). Recently, studies from our laboratory have demonstrated transactivation of EGFR resulting in activation of the MAPK (187). Mutation of the FP receptor in mice is associated aberrant parturition thus further confirming a role for FP mediated effects in reproduction (188).

1.15.3 IP Receptors

PGI_2 mediates its actions via the IP receptor. PGI_2 is the predominant prostaglandin produced by endothelial cells and its actions oppose those of TXA_2. The IP receptor can couple with G_s, G_q or G_i, which results in an elevation of cAMP, phosphoinositide turnover and an inhibition of cAMP respectively. However, coupling with G_s and activating cAMP turnover is the predominant signalling

pathway activated (148, 149). Alternative C terminal modifications allows for the coupling to multiple signalling pathways (148). The IP receptor undergoes agonist mediated desensitisation by the phosphorylation of the C terminal via a PKC dependent mechanism. Additionally, studies have demonstrated the internalisation of the IP receptor by a PKC independent process (148). IP receptor expression has been confirmed in the neurons of the dorsal root ganglia; including those that express substance P, indicating a role in the mediation of pain. In the kidney, IP receptors have demonstrated in the glomerulus suggesting an involvement in glomuerular filtration (144). Recently, IP receptors have been termporally localised in the human endometrium (189).

1.15.4 DP Receptors

PGD_2 exerts its functions via the DP receptors DP1 and the recently discovered CRTH2 (DP2) receptor (151, 190, 191). Previously, the DP receptor has been reported to be expressed at low levels in most tissues (149). PGD_2 mediated effects have been shown to play a role in a wide range of physiological and pathological conditions (190). PGD2 is known to be involved in the allergic response and have central nervous system actions; including an involvement in sleep (190). Activation of the DP receptor increases intracellular cAMP and Ca^2 levels (149). There is some evidence that signalling of the CRTH2 receptor is cell specific and may lead to alternative intracellular events in different cells (192). K562 cells (an erythroleukemia cell line) and Jurkat cells (a human leukaemia cell line) have been transfected with CRTH2. Activation of CRTH2 in K562 cells is associated with

increased Ca^{2+} levels. In contrast, activation of Jurkat cells was not accompanied with Ca^{2+} mobilisation (192). Moreover, various studies have reported that PGD_2 can signal via G_s, G_q, and G_i coupled receptors (151, 190).

1.15.5 TP Receptors

TXA_2 mediates its actions via the TP receptor which exists as multiple splice variants in humans; TPα and TPβ (193). The TP receptor is expressed in tissues supplied with a rich vasculature; such as the lung, kidney and heart, and also those related with immune function; including the spleen and thymus (144). The actions of thromboxane include contraction of smooth muscle and a mediator of platelet aggregation (149, 193). The TP receptor couples to a number of G proteins including G_q, G_s and G_i this results in activation of PLC-β and IP3 / DAG turnover, increased intracellular cAMP and reduced cAMP generation respectively (148).

1.16 PPAR Receptors

Mediation of prostaglandin actions via GPCRs is well accepted. The presence of COX-2 in the nuclear envelope has led to the possiblity that prostaglandins once synthesised may mediate effects via nuclear receptors. In addition to the nuclear localised EP receptors (160, 161), there is evidence that COX-2 products may elicit effects via the peroxisome proliferators-activated receptors (PPARs). The PPARs are nuclear located receptors. Three isoforms of the PPAR receptors have been

identified, namely, PPARα, PPARδ/β and PPARγ and these are closely homologous members of the nuclear hormone receptor superfamily (156).

Ligands for PPARs include fatty acids, prostaglandins, NSAIDs and anti-diabetic drugs (194). Upon activation by ligand binding, the receptors form a heterodimer with the retinoid X receptor and bind to PPAR responsive elements resulting in gene regulation. In addition, PPARγ has been demonstrated to block AP-1 and NFκβ transcription (195). PGJ_2 is a dehydration product of PGD_2 and the 15-deoxy metabolite of PGJ_2 (15d- PGJ_2) has been demonstrated to be a ligand for the PPARγ receptor and widely studied. PPARγ mediated effects are generally thought to be anti inflammatory and pro apoptotic (156). The dependence of 15d- PGJ_2 on the synthesis of PGD_2 has been hypothesised to result in delayed synthesis and therefore act in the resolution of inflammation (156). PPARγ ligands have been shown to inhibit the secretion of inflammatory mediators from mast cells (156). Inhibition of COX-2 expression and PGE_2 synthesis by PPARγ ligands was observed in human epithelial cells stimulated with phorbol 12-myristate 13-acetate (PMA) (196). In colon cancer cell lines and a mouse model of colonic inflammation, PPARγ ligands reduced cytokine production. These observations have led to the suggestion that PPARγ ligands may be of therapeutic use (156).

There is however some controversy surrounding PGJ_2 and whether it is produced endogenously and at high enough concentrations to activate PPARγ (157). Another metabolite of PGJ_2; Δ^{12}- PGJ_2 has been detected in urine and this has been taken by some as evidence of endogenous dehydration of PGD_2 to its metabolites (197). Since

NSAIDs can act as ligands for the PPAR receptors, this has further limited the study of the relationship between COX and PPAR.

1.17 Some Prostaglandin Elicited Actions in Physiology and Pathology

The involvement of prostaglandins in physiological and pathological states has been recognised for some time, recent prostanoid receptor knock out mice studies have helped to begin unravelling the molecular pathways that elicit prostanoid actions (see table 1.1) for a summary of these studies.

Genotypes	Phenotypes
DP (-/-)	Decreased allergic responses in ovalbumin-induced bronchial asthma
EP1 (-/-)	Decreased aberrant foci formation to azoxymethane
EP2 (-/-)	Impaired ovulation and fertilisation
	Salt-sensitive hypertension
	Vasopressor or impaired vasodepressor response to intravenous PGE_2
	Loss of bronchodilation with PGE_2
	Impaired osteoclastogenesis in vitro
EP3 (-/-)	Impaired febrile response to pyrogens
	Impaired duodenal bicarbonate secretion and mucosal integrity
	Enhanced vasodepressor response to intravenous infusion of PGE_2
	Disappearance of indomethacin-sensitive urine diluting function
EP4 (-/-)	Patent ductus arterosus
	Impaired vasodepressor response to intravenous infusion of PGE_2
	Decreased inflammation-dependent bone resorption
FP(-/-)	Loss of parturition
IP(-/-)	Thrombotic tendency
	Decreased inflammatory swelling
	Decreased acetic acid writhing
TP(-/-)	Bleeding tendency and resistance to thromboembolism

Table 1.1 Major phenotypes of prostanoid receptor knockout mice (reproduced from Kobayashi and Narumiya (198))

1.17.1 Reproduction

1.17.1.1 Uterine Function

The predominant prostaglandins synthesised in the endometrium are PGE_2 and $PGF_{2\alpha}$. Small amounts of TXA_2, PGD_2 and PGI_2 are also synthesised (4, 199). The prostanoids produced in the endometrium may have a number of roles. Prostanoids are thought to be important in vascular function. Both PGE_2 and PGI_2 are vasodilatory, conversely, $PGF_{2\alpha}$ and TXA_2 cause vasoconstriction (73). Furthermore, myometrial contractility is under the influence of prostanoids. PGE_2 and PGI_2 inhibit myometrial contractility whilst $PGF_{2\alpha}$ induces contractility (4, 199).

1.17.1.2 Ovulation

The importance of prostaglandins in normal ovulation is demonstrated by COX-2 knockout mice which display multiple reproductive failures including impaired ovulation (80). Lutenising Hormone (LH) promotes COX-2 expression and ultimately prostaglandin synthesis. Successful ovulation and rupture of the follicle depends in part on COX-2 induction (200).

1.17.1.3 Luteolysis and Parturition

In ewes, COX activity and PGF2α are both seen to increase prior to the onset of luteolysis (201). Disruption of the FP receptor in mice results in failed parturition

(202). PGF2α is an important mediator of luteolysis. It has been suggested that PGF2α may induce luteolysis through apoptosis of luteal cells. A correlation between FP mRNA expression and luteal cell apoptosis in pseudopregnancy has been demonstrated (202).

1.17.2 Gastrointestinal Function

The importance of prostaglandins in maintaining gastric integrity is partly demonstrated by epidemiological evidence that gastric ulcers and irritation are associated with long term NSAID use. The cytoprotective prostaglandins are thought to be predominantly derived from COX-1 activity (116). In a mouse model of induced gastric lesions with HCl/ethanol, the cytoprotective effect of taurocholate was reduced by indomethacin but not NS398. The same study demonstrated the protective effect mediated by prostaglandins was via the EP1 receptor (203). The importance of EP4 elicited effects in cytoprotection have also been reported (181). Radiation injury in mice results in reduced crypt stem cell number which is augmented with the non selective COX inhibitor indomethacin. The further reduction in crypt stem cell number is not observed with NS398, a non selective COX-2 inhibitor (73). The gastric actions of PGI_2 and PGE_2 are vasodilation, enhanced cytoprotective gastric fluid flow and inhibition of gastric acid production (204).

1.17.3 Renal Function

Prostaglandins are crucial for maintaining renal function (159). Prostaglandins demonstrate multiple roles within the kidney and this is highlighted by the expression of various prostaglandin receptors in the kidney (159). Prostaglandins are important in the control of rennin release, the regulation of vascular tone and the control of tubular function. PGE_2, PGI_2, $PGF_{2\alpha}$ and TXA_2 are all synthesised within the kidney (205). Whilst prostaglandins derived from COX-1 are thought to be important for preserving normal renal function, mice with a disrupted COX-2 gene and not COX-1 display renal dysfunction that leads to death (81). The relative importance and contribution of COX-2 derived prostaglandins in the human kidney has yet to be established.

1.17.4 CNS

Within the central nervous system, prostaglandins are involved in various systems including sleep, fever generation, and are implicated in the control of the autonomic nervous system and sensory processing (205). PGD_2 and PGE_2 are both found at high levels within the brain. PGD_2 and PGE_2 have opposing actions on sleep; PGD_2 induces sleep, conversely, PGE_2 reduces sleep (205).

Injection of PGE_2 intracerebroventricularly induces fever in wild type mice. NSAIDs such as aspirin and indomethacin reduce fever indicating a role for COX derived products in fever generation (206). Mice lacking the EP3 receptor do not display an

elevated temperature in response to PGE_2 or IL-1β; which is thought to mediate its fever inducing effects via PGE_2. This study suggests EP3 is important in mediating a febrile response (206).

1.18 COX-2 and Prostaglandin Mediated Effects That May be Involved in Endometrial Function

As described in section 1.3. The endometrium undergoes cyclical angiogenesis, apoptosis and proliferation with menstruation involving an inflammatory like mechanism. Numerous studies have demonstrated overexpression of COX-2 and the accompanied increased prostaglandin synthesis is associated with the promotion of angiogenesis, an inhibition of apoptosis, and enhanced cellular proliferation.

1.18.1 COX Enzymes and Prostaglandins in Angiogenesis

There have been many studies investigating the relationship between the COX enzymes and prostaglandins in angiogenesis. An in vitro model demonstrated co-culture of rat intestinal epithelial cells overexpressing COX-2 and endothelial cells results in enhanced synthesis of prostaglandins, and increases angiogenic factor expression such as VEGF (138, 207). Once synthesised, the angiogenic factors act in a paracrine manner to promote microvascular tube formation and endothelial cell migration (138). Overexpression of COX-1 in HeLa cells was associataed with upregulated angiogenic factors (60). Other in vitro studies have confirmed

upregulation of angiogenic factors and their receptors by prostaglandins such as PGE_2, PGI_2 and $PGF_{2\alpha}$ (168, 207-210). In a murine model of FAP, a direct relationship between COX-2, EP2 and angiogenic factor expression and microvessel density in intestinal polyps has been demonstrated. In the same study, EP2 knockout mice displayed a reduction in the size of the intestinal polyps concurrent with reduced COX-2, PGE_2 and angiogenic factor expression (171, 211). This study implicates the EP2 receptor in mediating the pro angiogenic actions of PGE_2. Other studies have shown EP3 and EP4 to be involved in angiogenesis (176, 212). PGE_2 may therefore elicit its angiogenic actions via multiple EP receptors. Recently the downstream signalling pathways activated by prostaglandins and linked to enhanced angiogenic factor secretion have been investigated. In Ishikawa cells, overexpression of EP2 or FP treatment with exogenous PGE_2 causes an enhanced secretion of VEGF by transactivation of the EGFR receptor and the ERK-1/2 pathways (168, 208). These studies demonstrate prostaglandins can act in an autocrine / paracrine manner to upregulate the synthesis of angiogenic factors and thus promote angiogenesis.

1.18.1.1 Antiangiogenic factors and Cathepsin D

In addition to proangiogenic factors, a number of endogenous antiangiogenic factors have also been identified including endostatin, angiostatin and thrombospondin-1 (213). It is thought that angiogenesis requires a balance of proangiogenic and antiangiogenic factors (214). In-vivo antiangiogenic factors are commonly proteolytic fragments of larger precursor proteins with distinct functions. Angiostatin and endostatin are cleaved fragments of plasminogen and collagen XVIII

respectively (213). The plasminogen structure contains 5 kringle domains (see figure 1.5). Cleavage of plasminogen at Arg^{560}-Val^{561} gives rise to proangiogenic plasmin (215). Alternative cleavage of plasminogen gives rise to antiangiogenic fragments. Kringles 1-3,(K1-K3), K1-K4; angiostatin and K5 all display antiangiogenic properties (215-217). Angiostatin has been demonstrated to be proteolytically derived from plasminogen by MMPs 3, 7, 9 and 12 and cathepsin D (218-220).

The cathepsins are lysosomal proteases with one of three active-site amino acids. Cathepsins B, C, H, F, K, L, O, S, V, and W are cysteine proteases. Cathepsins D and E have an aspartic residue and Cathepsin G a serine residue in the active site (221). The cathepsins exhibit high a high homology with the plant protease papain and display optimum activity at an acidic pH (222). The role of cathepsins is not limited to protein turnover. It has been demonstrated that cathepsins contribute to neovascularisation of endothelial cells, antigen presentation and cellular proliferation (221). Cathepsins D, B and L are thought to act collectively to promote a carcinogenic state (223). Studies have shown an enhanced expression of cathepsin D in estrogen receptor positive breast cancer (221). Furthermore, cathepsin D is thought to be a prognostic marker for reduced survival. Interestingly, in endometrial cancer, a reduced cathepsin D expression has been associated with a poor prognosis (224). To date overexpression of COX-2 and prostaglandins in the regulation of antiangiogenic factors has not been reported on.

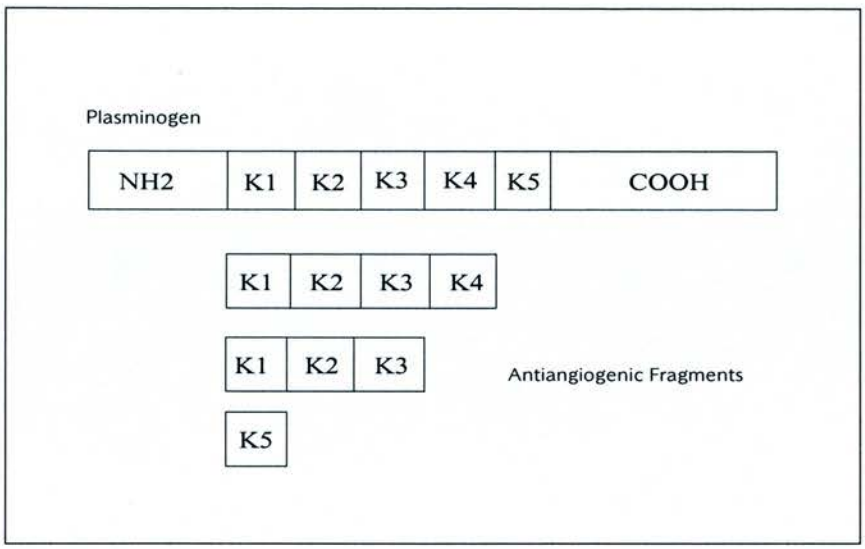

Figure 1.5 Schematic representation of the generation of antiangiogenic angiostatin fragments by the proteolytic action of agents such as cathepsin D on plasminogen.

1.18.2 Apoptosis

Apoptosis or programmed cell death is a key process in the shedding of the endometrium. There are a variety of pro and anti apoptotic genes and a balance of expression is important in maintaining normal tissue homeostasis (225). Apoptosis induced by members of the bcl-2 family of proteins is mediated by cytochrome C release from mitochondria (225). Upregulation of the anti apoptotic bcl-2 gene has been demonstrated in various tumours (225). Overexpression of COX-2 in epithelial cells has been shown to increase the expression of the bcl-2 protein and inhibit apoptosis. This effect was reversed with the administration of NSAIDs (137). Upregulated COX-2 is associated with a three fold increase in the duration of the G1 phase and reduced cyclin D1 which may contribute to the observed reduction in apoptosis (226). Treatment with NSAIDs can induce apoptosis as demonstrated in

patients with FAP and cervical cancers (227, 228). A further action of NSAIDs may be to inhibit Akt/PKB in the PI-3 kinase pathway. It is suggested that these actions are independent of COX-2 (229, 230). Mutations in tumour suppressor genes such as p53 are commonly seen in cancers including endometrial (44). An in vitro model has demonstrated overexpression of mutated p53 in mouse embryo fibroblasts results in enhanced COX-2 expression coincident with elevated PGE_2 secretion (231). Thus mutations in p53 may contribute to the overexpression of COX-2 observed in variety of tumours. Aberrant expression of bcl-2 has been described in endometriotic endometrial tissue. These studies suggest that inhibition of apoptosis mediated by COX-2 and prostaglandins may contribute to tumour progression and benign gynaecological pathologies.

1.18.3 Proliferation

Overexpression of COX-2 and the resultant elevation in prostaglandin synthesis has been demonstrated to promote cellular proliferation (137, 232, 233). The proliferative effects of the prostaglandins are mediated via diverse pathways. In endometrial tissue and cell lines, PGE_2 and $PGF_{2\alpha}$ have been shown to induce proliferation via the ERK-1/2 pathway and a PLC dependant pathway respectively (183, 234). Of the four PGE_2 receptors, the HES cell line; an endometrial epithelial cell line, only expresses EP4. It has been suggested that in this that PGE_2 mediated proliferative effect is therefore via EP4 signalling (234). Treatment of cells with NSAIDs has been shown to inhibit cellular proliferation. (235-237). Epidermal growth factor induces COX-2 expression and this is concomitant with enhanced

mitogenesis. Inhibition of either epidermal growth factor or COX-2 reduces mitogenesis (237). PGE_2 has been demonstrated to induce COX-2 expression via the activation of the Ras MAPK pathway (238). It was suggested that the proliferative action of PGE_2 is mediated in part by upregulating COX-2 and therefore initiating a feed forward mechanism (238). Thus within the endometrium, PGE_2 and $PGF_{2\alpha}$ may be important in cellular proliferation and re-growth of the endometrium post menstruation.

1.19 Aims and Objectives of Thesis:

As detailed in section 1.6.1, excessive blood loss during menstruation (mennorhagia), dysmenorrhoea often associated with painful menstruation and endometriosis are common benign uterine pathologies. These gynaecological complaints are considerable reproductive health problems for women and also place a financial burden on healthcare. Research into the underlining molecular mechanisms of these conditions could lead to improved and less invasive therapies.

The molecular mechanisms of these gynaecological conditions are poorly understood. However, a role for cyclooxygenase enzymes and its products the prostaglandins (such as prostaglandin E_2) has been observed. Elevated binding sites for prostaglandins and an altered ratio of prostaglandins synthesised in endometrium from women with benign conditions as mentioned above relative to those from normal endometrium have previously been described. Moreover, first line treatment for menorrhagia and dysmenorrhoea is often the use of NSAIDs which act by inhibiting the COX enzymes. However the role of COX and its products in the human endometrium has yet to be elucidated.

As described in sections 1.6.1 and 1.13, COX-2 and its products the prostaglandins have been associated with a range of gynaecological conditions and implicated in promoting angiogenesis within a tumour environment. This thesis examines the hypothesis that COX-2 and its products the prostaglandins may play a role in the promotion of vascular function within the human endometrium.

The specific aims of the research were to:

1) Investigate the temporal expression and signalling of the prostaglandin E_2 pathway in the normal human endometrium across the menstrual cycle. This was determined using a variety of techniques in endometrial tissue samples collected from across the menstrual cycle as described in chapter 3.

2) Investigate the role of cyclooxygenase-2 and prostaglandin E_2 in Ishikawa cells, an endometrial epithelial cell line. This was achieved by stably transfecting Ishikawa cells with cyclooxygenase-2 cDNA in either the sense or antisense directions and determining prostaglandin synthesis, receptor expression and signalling as detailed in chapter 4.

3) To investigate the potential role of cyclooxygenase-2 in regulation of endometrial angiogenesis. This was investigated by cDNA array technology in combination with in vitro studies using the Ishikawa COX-2 stable cell line outlined in chapter 4.

Chapter 2

General Methods

Materials and Methods

2.1 Reagents and Suppliers

All reagents unless otherwise specified were purchased from Sigma Chemical Company (Dorset, UK). Ishikawa cells were purchased from European Collection of Cell Culture. Dulbecco's modified Eagle's medium nutrient mixture F-12 was purchased from Life Technologies (Gibco, Life Technologies Ltd, Paisly, UK), Foetal calf serum and penicillin-streptomycin was purchased from PAA (PAA Laborotaries Ltd., Yeovil, UK). The following antibodies used for Western Blotting were purchased from Santa Cruz Biotechnology, inc. (Autogenbioclear, Wiltshire, UK): COX-1 goat polyclonal (sc-1752), COX-2 goat polyclonal (sc-12745), cathepsin D goat polyclonal (sc-6486), plasminogen (sc-15034), β-actin (sc-1616). Anti-goat-alkaline phosphatase antibody was purchased from Sigma (Sigma Chemical Company, Dorset, UK). The EP2 and EP4 antibodies used for immunohistochemistry were purchased from (Caymen). Cloning cylinders, G418 and indomethacin were purchased from Sigma (Sigma Chemical Company, Dorset, UK). NS398 was purchased from Calbiochem (Calbiochem, Beeston, Nottingham, UK). The ECF chemiluminescence system was purchased from Amersham (Amersham, Little Chalfont, Bucks, UK). Enzymes were purchased from Boehringer Mannheim (Buckinghamshire, UK) or Promega (Southampton, UK)

2.2 Endometrial Tissue Collection

Ethical approval was obtained from the Lothian research ethics committee and written informed consent was obtained from all the patients before tissue collection. Endometrial biopsies were collected using an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France) from women with regular menstrual cycles at different stages of the menstrual cycle see section 3.2. Full thickness biopsies were collected across the menstrual cycle from women undergoing hysterectomies for benign gynaecological indications. Once collected, the tissue, was either snap frozen on dry ice and stored at -70°C for RNA extraction, fixed in neutral buffered formalin, and wax imbedded for immunohistochemical analyses or placed in RPMI (containing 2 mmol/ litre L-glutamine, 100 U penicillin and 100μg/ml streptomycin) for *in vitro* culture. All subjects reported regular menstrual cycles (cycle length 25-35 days) and no women had received a hormonal preparation in the 3 months preceding biopsy collection. Biopsies were dated according to stated last menstrual period and were confirmed by histological assessment according to criteria of Noyes et al (15). Furthermore, circulating oestradiol and progesterone concentrations at the time of biopsy were consistent for both stated last menstrual period and histological assignment of menstrual cycle stage.

2.3 Cell Culture

General sterile cell culture techniques were employed. Unless otherwise stated, Ishikawa cells were grown in culture media supplemented with 10% Fetal Calf

Serum (FCS) and 1% Penicillin/ Streptomycin and maintained at 37°C, 5% CO_2. The cells were generally passaged twice a week. Once the cells reached full confluency, the cells were washed twice with PBS, rinsed briefly with trypsin and then placed in the incubator for 5 minutes allowing the cells to detach from the flask. The cells were resuspended in 5ml of culture media and 1ml was transferred to a new 75ml flask with 14ml of media and maintained as the stock. The remaining 4mls of cell suspension was used to seed out fresh experiments.

2.4 Protein

2.4 1 Protein Extraction From Cells

Cells were grown to the desired confluency as indicated in section 4.2 in 6 well plates, the media was aspirated and the cells were washed twice with PBS, 2mls of serum free media was then added to each well to synchronise the cells overnight. Following overnight starvation, the cells were washed twice with ice cold PBS and lysed on ice in 200µl buffer (containing 150mM NaCL, 50mM Tris pH 7.4, 10mM EDTA, 0.6% NP40, 1mM Na3VO4, 10% glycerol, 10µg/ml Pepstatin, 1mM PMSF) for 5 minutes. The wells were scraped using a clean plastic cell scraper, this was followed by repeated pipetting up and down of the cell suspension to ensure complete lysis. The lysates were clarified by centrifugation at 14000g for 15 minutes at 4°C to pellet cell debris. The supernatants were collected by pipette and stored at -70°C in fresh tubes until further use.

2.4 2 Protein Quantification

Protein concentrations were quantified using the modified Lowry method (Bio-Rad D2 protein Assay kit; Bio-Rad Laboratories, Hemel Hempstead, UK). A standard curve using bovine serum albumin (BSA, Promega,Southampton, UK) was constructed. BSA was diluted in water and 1:50 lysis buffer to a concentration of 200µg. The standard (200µg/ml) was then serially diluted 6 times in water and 1:50 lysis buffer. The standards at concentrations of 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml and 0µg/ml were used to construct the standard curve. Samples were diluted 1:50 in water. The standards and samples (25µl) were pipetted to wells in duplicate on a 96 well plate. To this, 25µl reagent A (+ 20 µl reagent S per ml reagent A used; supplied with the kit) followed by 100µl reagent B were added. The plate was incubated at room temperature to allow for colour development for approximately 15 minutes. The absorbance was measured at 690nm using a Multiscan® MCC/340 plate reader. Average protein concentrations of samples were determined from the standard curve constructed by extrapolation using the Assay Zap software (Biosoft).

2.4 3 SDS-PAGE

SDS-PAGE was performed using pre-cast gels with a gradient of 4-20% Tris Glycine, with 15 wells (Novex Invitrogen). Unless otherwise stated 15µg of protein sample (extracted and quantified as described above) was used for SDS-PAGE. The 15µg samples were pipetted into tubes and diluted in distilled H_20 (dH_20) to a

volume of 20µl and a further 5µl of 5X protein loading buffer (1mM Tris pH6.8, 50% glycerol, 1% SDS, 12.5% β Mercaptoethanol, 0.01% Bromo phenol Blue) was added to each sample. Thus giving a sample volume of 25µl to be transferred to each well on the gel. Prior to loading on the gel, the samples were boiled at 95°C for 5 minutes using a pre-heated hot block. Before use, the wells of the pre-cast cassettes were marked with pen on the outside of the cassettes to aid loading the gel, the cassettes were then gently rinsed with dH_20 following the removal of the plastic comb. Once the gels were set up in the gel tank according to the manufacturers instructions, running buffer (25mM Tris-HCL, 0.2M glycine, 0.1% SDS) was poured in between the two gels and the pre prepared samples were loaded into the individual wells. 12µl of the pre stained See Blue protein marker (Novex) was loaded to the first well on each gel. The migration of proteins was then monitored along side the protein molecular marker. The gels were run at a constant 40mA per gel for approximately 90 minutes. The pre cast gels were cracked open using a palette knife and the gels transferred to a clean glass plate. The gel wells were then cut off and discarded in preparation for immunoblotting as described below.

2.4 4 Western Blotting

Following electrophoresis, the proteins were transferred from the gel to PVDF membrane. The pre cut PVDF membrane was prepared by initially placing it in 100% Methanol for 30s, transferring it to dH20 for 1 minute to reduce its hydrophobicity and finally in transfer buffer (25 mM Tris/HCl, 0.192 M glycine, 20% methanol) for 2 minutes. The gel and PVDF membrane were sandwiched

between 8 pieces of Whatman paper pre cut to the same size as the gel and PVDF membrane; four on top and four underneath, which had also been equilibrated in the transfer buffer for 2 minutes. The sandwich of gel, PVDF and Whatman paper was placed on the blotter and any air bubbles were removed by rolling a clean pasteur pipette over the stack. Using a semi dry blotter (BIO-RAD), proteins were transferred for 1hr at a constant 12V. Following immunoblotting, the PVDF membrane was immersed in 100% methanol for 30s to restore its hydrophobicity and allowed to dry for 15 minutes. The membrane was then blocked in TNS Tween (50mM Tris-HCl, 150mM NaCl and 0.05% (v/v) Tween 20) containing 5% skimmed milk powder to bind to any unbound protein sites for 1hr at room temperature on a shaker. The membrane was then incubated on a shaker over night at 4°C with the primary antibody of interest; diluted in TNS Tween 5% fat free milk powder. Following this incubation the membrane was washed 3 times with TBS Tween for 10 minutes at room temperature on the shaker. The membrane was then incubated with the relevant secondary antibody linked to alkaline phosphatase diluted in TBS Tween 5% skimmed milk powder for 1hr at room temperature on the shaker. Finally the membrane was then washed 3 times as above. Following the manufactures instructions, the membrane was revealed using the ECF chemiluminessence system. ECF solution was pippetted onto the membrane. The membrane was left in the dark for 10 minutes, following this incubation, the ECF solution was removed from the membrane by allowing the solution to run off the membrane onto Whatman paper. The membrane was then left to dry in the dark for a further 15 minutes and then visualised by phosphorimager analysis. Proteins were semi quantified by densitometry using STORM 860 system (Molecular Dynamics, Amersham

Biosciences, Buckinghamshire, United Kingdom). The molecular weight of the protein bands observed were determined by comparing with the mobility of the SeeBlue standard.

2.5 RNA

2.5.1 RNA Extraction From Cells and Endometrial Tissue

2.5.1 1 Cells

Cells were grown to the desired confluency in 6 well plates (as described in chapter 4.2 the media was aspirated from the wells and the wells were washed twice with PBS. Serum free media (2ml) was then added to each well and the plates were incubated overnight to synchronise the cells. Following the starvation, the wells containing the cells were washed twice with cold PBS. RNA was then extracted from cells using Tri Reagent (Sigma). Tri Reagent 1ml was added to each well and repeated pipetting up and down ensured that complete dissociation occurred. The plates were allowed to stand for 10 minutes at room temperature and the contents of the wells were transferred to RNase free tubes.

2.5.1.2 Endometrial Tissue

RNA was extracted from endometrial tissue also using Tri Reagent, according to the manufacturers instructions. Endometrial tissue was homogenised using an Ultra-

Turrak T8 homogeniser (IKA Labortechnik) for approximately 1 minute in 1ml of Tri Reagent per 100 mg of tissue and allowed to stand for 10 minutes at room temperature.

For both cells and endometrial tissue, chloroform (200µl per 1ml Tri Reagent) was added to each tube. The tubes were shaken vigorously for 15 seconds and allowed to stand at room temperature for a further 10 minutes. Following this, the tubes were centrifuged at 14 000 X g for 20 minutes at 4°C. The aqueous supernatant containing the RNA was transferred to fresh RNase free tubes and 500µl isopropanol /ml Tri Reagent used was added to precipitate the RNA. The tubes were inverted and allowed to stand for 10 minutes at room temperature before centrifugation at 14 000 x g for 20 minutes at 4°C. The supernatant was discarded and 1ml 75% ethanol in DEPC water /ml Tri Reagent used was added to each tube. The tubes were vortexed briefly to wash the RNA pellet and then centrifuged for 10 minutes at 10 000 x g, 4°C. The supernatants were discarded and the tubes were inverted and left to stand on a RNAse free surface to allow the pellets to dry. The RNA was then resuspended in 25µl DEPC water by warming the tubes at 65°C for 5 minutes followed by repeated pipetting up and down. The resuspended RNA was then transferred to fresh RNAse free tubes and stored at -70°C for quantification.

2.5.2 RNA and DNA Quantification and Quality determination

RNA samples were diluted 1:10 in DEPC water and the absorbance at 260 and 280nM was measured using a spectrophotometer. The concentration of RNA or

DNA for each sample was determined using the Beer Lambert law A=εbc (where A is the absorbance, ε is the molar absorbtivity, b is the path lenth of the sample and c is the concentration of the compound (RNA) in the solution). Assuming the relationship of an optical density (OD) absorbance reading of 1 at 260nM = 40 μg/ml RNA and 50 μg/ml for double stranded DNA the concentration of RNA was calculated as follows. The 260nM absorbance reading was multiplied by 40μg/ml and then 10 to allow for the dilution factor. The samples were considered pure with limited protein contamination when the ratio of the absorbance readings at 260nM and 280nM was greater than 1.69. The ratio was obtained by dividing the reading at 260nM by the absorbance reading at 280nM

2.5.3 Reverse Transcription

Prior to reverse transcription, the RNA samples were DNAse I treated (Gibco) according to the manufacturers instructions. RNA (1μg) samples was added to DNAse reaction buffer (1μl), DNAse I (1U) made up to a final volume of 10μl and briefly vortexed. The samples were incubated at room temperature for 15 minutes. The DNAse I was inactivated by the addition of EDTA (1μl 25mM) followed by 10 minutes incubation at 65°C on a pre-heated hot block. RNA extracted from tissue or cells was reversed transcribed using a mix of 5.5mM $MgCl_2$, 0.5mM each deoxynucleotide triphosphates, 2.5μM random hexamers, ribonuclease inhibitor (0.4 U /ml) and 1.25 U/ml Multiscribe reverse transcriptase (TaqMan GeneAMP RNA PCR kit, PE Applied Biosystems, Warrington, UK). A fresh reverse transcription mix was made prior to each reverse transcription reaction. Template RNA (400ng)

was added to reverse transcription mix aliquoted in separate tubes (16µl) to a final volume of 20µl. A tube containing no reverse transcriptase and one with no template were used as negative controls to confirm there was no genomic DNA contamination. A tube containing RNA from a late secretory endometrial tissue sample was used as a positive control. Following gentle mixing of the tubes using a vortex, the tubes were incubated as follows, 60 minutes at 25°C, 45 minutes at 48°C and 95°C for 5 minutes. The transcribed cDNA was then stored at –20°C until further use.

2.5.4 Polymerase Chain Reaction (PCR)

PCR is a technique that allows the amplification of a DNA sequence of interest that lies between two known sequences. The principles of PCR are as follows. In the presence of deoxynucleotide triphosphates (dNTPs), magnesium and reaction buffer, 5' and 3' primers with sequences complementary to the region that lies at the end of the DNA sequence of interest are extended by the action of DNA polymerase. The PCR reaction occurs when the samples are placed through a cycle of different temperatures. Initially, the double stranded DNA is heated to 95°C to denature the DNA and produce single stranded DNA. Following denaturing, the reaction temperature is reduced to between 40°C and 60°C to allow for the primers to anneal to the single stranded DNA. Finally, DNA synthesis occurs when the temperature is increased to the optimum temperature for DNA polymerase to extend the primers. Theoretically, the target sequence of interest is doubled after each PCR cycle. Real Time Quantitative PCR enables the quantification of a sequence of interest. In

addition to the reaction mixture outlined above for a PCR reaction, a probe containing a reporter dye at the 5' end and a quencher dye at the 3' end is introduced. In the presence of the sequence of interest, the probe anneals in between the forward and reverse primers, the 5' 3' nuclease activity of the Taq DNA polymerase cleaves the probe. Separation of the reporter dye from the quencher results in a measurable increase in fluorescence. An increase in fluorescence detected is indicative of an elevation in the PCR product of interest.

2.5.5 Real Time Quantitative PCR

To investigate the RNA expression of the prostaglandin EP receptors, real time Quantitative PCR was utilised. Real time quantitative PCR was performed using a PCR mix which consisted of 1X mastermix, forward and reverse primers for the sequence of interest, (300nM), the relevant probe (200nM) and ribosomal 18s forward, reverse and probe (50nM all from PE Applied Biosystems). 48µl of PCR reaction mix was aliquoted to separate tubes and 2µl (40µg) of reverse transcribed cDNA was added and gently mixed by vortex. Samples (24µl) were pipetted in duplicate into wells on a PCR plate along with the positive and negative controls from the reverse transcription reaction. Furthermore, a negative control for the PCR containing 2µl H_20 and no cDNA was also pipetted in duplicate on the PCR plate. The wells were sealed using optical lids and the PCR was carried out using an ABI Prism 7700 (PE Applied Biosystems). 18s rRNA was used as an internal standard to normalise the samples to RNA loading. Results were expressed relative to the positive standard run on each PCR.

Gene		Primers and Probe
COX-2	Forward Primer	5' - CCT TCC TCC TGT GCC TGA TG – 3'
	Reverse Primer	5' – ACA ATC TCA TTT GAA TCA GGA AGC T - 3'
	FAM linked Probe	5' TGC CCG ACT CCC TTG GGT GTC A - 3'
EP1	Forward Primer	5' - AGA TGG TGG GCC AGC TTG T - 3'
	Reverse Primer	5' - GCC ACC AAC ACC AGC ATT G - 3'
	FAM linked Probe	5' - CAG CAG ATG CAC GAC ACC ACC ATG - 3'
EP2	Forward Primer	5'- GAC CGT TTA CCT GCA GCT GTA - 3'
	Reverse Primer	5' - TGA AGT TGC ACC CGA GCA - 3'
	FAM linked Probe	5' - CCA CCC TGC TGC TGC TTC TCA TTG TCT - 3'
EP3	Forward Primer	5' - GAC GGC CAT TCA GCT TAT GG - 3'
	Reverse Primer	5' - TTG AAG ATC ATT TTC AAC ATC ATT ATC A - 3'
	FAM linked Probe	5' - CTG TCG GTC TGC TGG TCT CCG CTC - 3'
EP4	Forward Primer	5' - ACG CCG CCT ACT CCT ACA TG - 3'
	Reverse Primer	5' - AGA GGA CGG TGG CGA GAA T - 3'
	FAM linked Probe	5' - ACG CGG GCT TCA GCT CCT TCC T - 3'
FP	Forward Primer	5' - GCA GCT GCG CTT CTT TCA A - 3'
	Reverse Primer	5' - CAC TGT CAT GAA GAT TAC TGA AAA AAA TAC-3'
	FAM linked Probe	5' - CAC AAC CTG CCA GAC GGA AAA CCG - 3'
18s	Forward Primer	5' - CGG CTA CCA CAT CCA AGG AA - 3'
	Reverse Primer	5' - GCT GGA ATT ACC GCG GCT - 3'
	VIC linked Probe	5' - TGC TGG CAC CAG ACT TGC CCT C - 3'

Table 2.1 Sequences for primers and probes used for quantitative RT PCR

2.6 cAMP Turnover

cAMP turnover in response to exogenous PGE_2 was measured in endometrial tissue, Ishikawa wild type, COX-2 sense and COX-2 antisense cells, for descriptions of the preparation and treatment of the tissue and the cells see sections 3.2.3 and 4.2.9. cAMP turnover was quantified using a cAMP competitive ELISA kit (Biomol, Affiniti, Exeter, UK) according to the manufacturer's instructions and normalized to

protein concentrations of the homogenate. Briefly, the assay was performed in a 96 well goat anti-rabbit IgG coated microtiter plate. The cAMP standard curve was constructed as instructed by the manufacturer. The standard was diluted four times in a ratio of 1:4 in HCl (0.1M). The standards at concentrations of 200 pmol/ml, 50 pmol/ml, 12.5 pmol/ml, 3.13 pmol/ml and 0.78 pmol/ml were used to construct the standard curve. The standards and samples (100µl) were pipetted into the 96 well plate. The alkaline phosphatase-cAMP conjugate and polyclonal rabbit-cAMP antibody were then added to the wells (50µl of each solution). The plate was then incubated at room temperature for 2 hours on a plate shaker at approximately 500 rpm. Following this, the contents of the plate was aspirated and the plate washed three times with the wash buffer provided (TBST containing sodium azide). To develop the assay, the conjugate and p-Npp (p-Nitrophenyl Phosphate, Disodium Salt) substrate were added to the wells (5µl and 200µl respectively) and the plate incubated at room temperature for 1 hour. The reaction was quenched with 50µl of stop solution (trisodium phosphate in water) provided with the kit. The absorbance was measured at 405 nm using a Multiscan® MCC/340 plate reader. Average cAMP concentrations of samples were determined from the standard curve constructed by extrapolation using the Assay Zap software (Biosoft). Protein concentrations were quantified using the modified Lowry method (Bio-Rad D2 protein Assay kit; Bio-Rad Laboratories, Hemel Hempstead, UK) as described in section 2.4.2.

2.7 Statistical Analysis

Statistical analysis of the data in this study was carried out by ANOVA using StatView 5.0 (Abacus Concepts, Berkeley, CA).

Chapter 3

PGE_2 and EP2/EP4 Receptor expression and signalling in the human Endometrium

3.1 Introduction

Two predominant isoforms of the cyclooxygenase enzymes have been identified (73). COX-1 is constitutively expressed, classically associated with physiological function and has recently been shown to be inducible in certain cancers (65, 90-92). COX-2 is the readily inducible form of the enzyme and is commonly associated with inflammation and pathological conditions including tumorigenesis (73, 79, 94). The COX enzymes catalyse the conversion of arachidonic acid to PGH_2 which is ultimately converted to prostanoids. There are five endogenous prostanoids namely PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane A2(149). Arachidonic acid, once released from the membrane phospholipids is converted to the prostanoid intermediate PGH2 by the COX enzymes. PGH2 acts as a substrate for the synthases specific to each prostanoid such as PGE synthase (PGES) for PGE_2 (239, 240). A number of distinct isoforms of PGES have currently been described. These include mPGES-1 and mPGES-2 which are both membrane bound, cPGES is the cytosolic isoform and GST-μ a glutathione S-transferase isoform and these have been shown to preferentially couple with either COX-1 or COX-2 (70). Once synthesized, prostaglandins mediate their effects via prostaglandin specific G protein coupled receptors. For instance, PGE_2 elicits its effects via its seven trans-membrane G protein-coupled receptors, of which four have been identified (EP1, EP2, EP3 and EP4). These receptors signal via alternate and in some cases opposing signaling pathways (149, 154). EP1 receptor activation leads to elevated insositol-tris-phosphate and Ca2+ levels, activation of both EP2 and EP4 results in increased

intracellular cAMP levels and depending on the splice variant, EP3 activation either decreases or increases cAMP levels (149).

Recent studies have demonstrated a role for COX enzymes and prostaglandins (PGs) in the regulation of epithelial cell growth and angiogenesis. In vivo and in vitro studies in epithelial cells, including human glandular endometrial Ishikawa cells, have demonstrated a role for COX-2 and its products such as PGE_2 in epithelial cell function. In those studies, elevated COX-2 and prostanoids, including PGE_2 have been shown to facilitate enhanced cellular proliferation, reduce apoptosis and increase the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) (65, 137, 138, 183, 207, 209, 211, 233, 234). Once synthesised, the angiogenic factors act in a paracrine manner to promote endothelial cell migration and microvascular tube formation (138). In the human endometrium, COX enzyme expression has been localised to epithelial and perivascular cells. Furthermore, PGE_2 synthesis and COX expression are maximal during the menstrual and proliferative phases (4, 23, 102, 241, 242). Recent studies have reported the temporal expression and signalling of two COX product receptors: $PGF_{2\alpha}$ receptor, FP and the IP_2 receptor, IP in the human endometrium (183, 189).

The aim of this study was to investigate the temporal expression and signalling of the prostaglandin E_2 pathway in the normal human endometrium across the menstrual cycle. mPGES-1 and PGE_2 were localised using immunohistochemical techniques (Peformed by Dr S. Milne). The expression of two PGE_2 receptors, namely EP2 and EP4, were localised and quantified using immunohistochemistry and quantitative RT

PCR respectively. Finally the functional signalling of these receptors in response to exogenous PGE_2 was determined by measuring the generation of cAMP using an ELISA.

3.2 Materials and Methods

3.2.1 Immunohistochemistry

The site of mPGES-1, PGE_2, EP2 receptor and EP4 receptor expression was localised in endometrial sections (5μM) by immunohistochemistry from across the menstrual cycle. For PGE_2 and mPGES-1, n=9 proliferative phase and n=9 secretory phase, EP2 and EP4 localisation; n=6 proliferative phase and n=6 secretory phase). Tissue was collected as described in section 2.2 and mounted onto glass slides. Sections were de-waxed in xylene (2 x 5 mins) and then re-hydrated in graded ethanol for 20 seconds at each concentration as follows: 100% Ethanol, 95% Ethanol and 70% Ethanol. The sections were then rinsed in water. An antigen retrieval step was carried out for mPGES-1, EP2 and EP4 localisation. For antigen retrieval, the slides were pressure cooked in sodium citrate buffer (0.01M , pH 6.0) for 5 minutes. Following pressure cooking, the slides were left to cool to room temperature for 20 minutes before rinsing in water until the slides were cool. Unless otherwise stated, all washes of the tissue samples were performed on a rocker for 5 minutes. All slides were then washed in Tris buffered Saline (TBS; Tris-HCl (50 mM), NaCl (150mM), pH 7.4). A peroxidase detection system was used for immunodetection therefore in order to block endogenous endoperoxidase activity, the slides were incubated with

3% H_2O_2 in methanol for 20 minutes at room temperature on a rocker. The tissue samples were washed in TBS prior to being blocked with swine serum (10% in TBS and 5% BSA) for 30 minutes at 25°C. The normal serum was removed by draining the slide on paper tissue, and any excess surrounding the tissue was wiped away. The slides were covered with Gelbond film (Flowgen, Rockland, ME, USA), hydrophobic side down and incubated overnight in a humidified chamber at 4°C with the primary antibody (either PGE_2 or mPGES-1 or EP2 or EP4). The rabbit anti - PGE_2 antibody (kindly supplied by Professor R W Kelly, MRC Human Reproductive Sciences Unit, Edinburgh, UK) at a dilution of 1:100, rabbit anti - PGE synthase, rabbit anti- EP2 and rabbit anti EP4 antibodies were used at a dilution of 1:250 (Caymen Chemicals). The antibodies were diluted in normal swine serum (10% in TBS) and BSA (5%). Preabsorption of the antibody with PGE synthase blocking peptide (at a ratio of antibody to peptide 1:10) was used for the mPGES-1 negative control, preabsorption of the antibody with PGE_2 was the PGE_2 negative control and normal rabbit IgG was used for the EP2 and EP4 controls. All the negative controls demonstrated negligible immunoreactivity. Following overnight incubation with the primary antibodies, the tissue sections were washed twice in TBS. The slides were then probed with swine anti-rabbit secondary IgG (Dako) for 30 minutes, 1:500 dilution (in normal swine serum (10% in TBS) and BSA (5%)) at 25°C. The tissue sections were washed twice in TBS and then incubated for 30 minutes with the pre-prepared streptavadin peroxidase complex (Dako). The peroxidase solution was prepared according to the manufacturers instructions in TBS (50 mM, pH 7.6; no NaCl) and at least 20 minutes before use. Following this, the slides were washed twice. To visualise bound antibody, the tissue sections were incubated with 3,3-

diaminobenzidine solution (DAB) prepared according to the manufacturers instructions (Dako) for approximately 10 minutes. The colour development was monitored microscopically and the reaction was quenched by washing in water. The tissue sections were then counterstained and mounted as follows. The slides were counterstained in haematoxylin for between 30 seconds and 4 minutes. Following a brief wash in water, the sections were rinsed briefly in acid alcohol again washed in water and then blued in Scott's tap water. Following a final wash in water, the slides were dehydrated in graded alcohol washes at 70% ethanol, 95% ethanol and 100% ethanol each for 5 minutes. Prior to being coverslipped with pertex (Cellpath, Hemel Hempstead, UK) the slides were cleared in histoclear and xylene each for 5 minutes.

3.2.2 Quantitative RT PCR

To investigate the expression of EP2 and EP4 receptors in the human endometrium, quantitative RT PCR was performed as described in section 2.5.5. Tissue samples were collected from across the menstrual cycle (n=9; Mid Proliferative, n=5; Late proliferative, n=7; early secretory, n=6; mid secretory, n=6 late secretory). Briefly, the RNA was extracted from the tissue samples using Tri Reagent (section 2.5.1.2). The RNA was reverse transcribed as described in section 2.5.3. The cDNA expression was measured by real time quantitative RT PCR. Data were analysed using Sequence Detector version 1.63 (Applied Biosystems) according to the manufacturer's instruction.

3.2.3 cAMP Measurement

Endometrial biopsies from across the menstrual cycle (n=6 from each of the following phases; proliferative, early and midsecretory phase) were finely minced with scissors and incubated in Petri dishes for 1.5 hours in RPMI media (2ml) containing 10% FCS, 2 mmol/liter L-glutamine, 100 IU penicillin, 100 μg streptomycin and 3 μg/ml indomethacin at 37°C in a humidified 5% CO_2 incubator. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was added to the tissue media to a final concentration of 1mM for a further 30 minutes at 37°C prior to treatment with PGE_2. PGE_2 (300nM) was added to the tissue culture media for 5 minutes. The control samples were treated the same but received no PGE_2. The tissue samples were harvested by centrifugation at 2000 x g. The supernatant was discarded by pipette and the tissue homogenised in 10ml HCl (0.1M) per gram of tissue. The cAMP concentration was determined as described in section 2.6 and was normalised to protein concentration determined as described in section 2.4.2.

3.3 Results

3.3.1 Expression of mPGES-1 and PGE_2 Synthesis in Endometrial Tissue

Membrane bound Prostaglandin E Synthase-1 (mPGES-1) was spatially and temporally localised in the endometrium by immunohistochemistry (see figure 3.1). mPGES-1 immunoreactivity was shown to vary according to the uterine region and stage of the menstrual cycle. mPGES-1 staining was demonstrated in the glandular epithelial cells (G) and endothelial cells (arrowed) of both the basalis and functionalis layers of the endometrium. Stromal staining of mPGES-1 was predominantly seen in the functionalis layer as opposed to the basalis layer where little immunoreactivity in the stromal cells was detected. The stromal immunoreactivity appeared to be temporally expressed with reduced staining in the late secretory phase (e) compared with other stages of the cycle. mPGES-1 was also localised to the myometrium with staining only visible in the endothelial cells. Viewed as a cross section (f), mPGES-1 expression was demonstrated in endothelial and glandular epithelial cells across the uterine section. Strong immunoreactivity in the stromal region of the functionalis layer was shown; with a reduction in stromal staining across the uterine section with limited staining in the myometrial fibroblasts.

PGE_2 synthesis in the human uterus was localised by immunohistochemistry (see figure 3.2). Spacial and temporal expression of PGE_2 in the uterus was demonstrated to show a similar pattern of immunoreactivity to mPGES-1. PGE_2 synthesis in the

glandular epithelial cells (G) and endothelial cells (arroweed) was detected in both the basalis (a) and functionalis (b) layers of the endometrium. Limited stromal staining was detected in the basalis layer, conversely in the functionalis layer, there was strong staining in the stromal region. Likewise with mPGES-1, there is a marked reduction in PGE_2 immunoreactivity in the stromal region during the late secretory phase (e) compared with other stages of the menstrual cycle. However, PGE_2 expression in the glandular and endothelial cells remains similar throughout the menstrual cycle. Full thickness endometrial biopsies (f) demonstrate a gradient in PGE_2 expression with maximal immunoreactivity in the functionalis layer in multiple cell types and limited PGE_2 synthesis detection in the myometrium in only the endothelial cells.

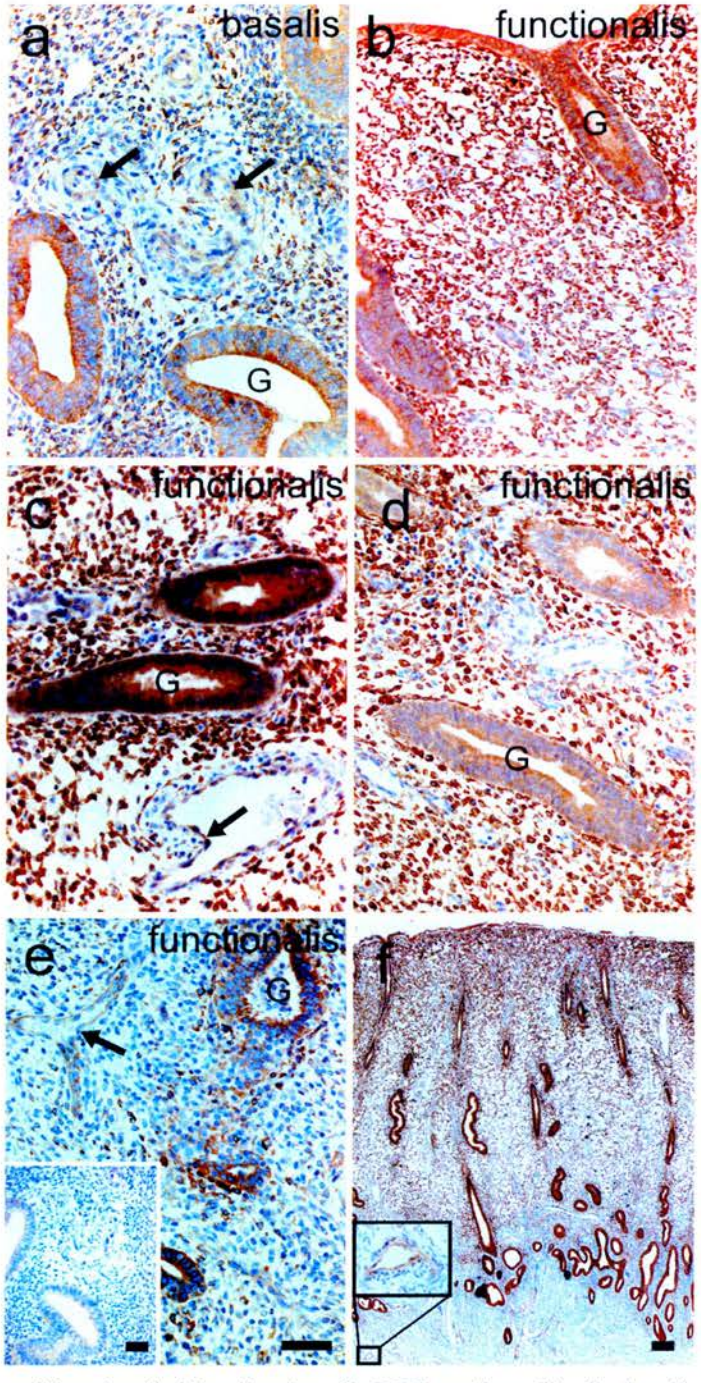

Figure 3.1 Immunohistochemical localisation of mPGE synthase-1 in the basalis and functionalis regions of the human endometrium. Lower PGE synthase immunoreactivity was detected in the stromal compartment of the basalis than in the functionalis region (a and b are the basalis and functionalis regions, respectively, of endometrial tissue collected during the midproliferative phase). In the functionalis region, PGE synthase immunoreactivity was detected at all stages of the menstrual cycle and was localised to glandular epithelial (G), stromal and endothelial (denoted by arrows) cells (c, late proliferative; d, early secretory; e, late secretory). Inset in e, A section that was stained with preadsorbed PGE synthase antibody (negative control). F, Full thickness uterine tissue, collected during the late proliferative phase, demonstrating spatial changes in PGE synthase immunoreactivity between basalis and functionalis regions of the endometrium and the myometrium. Inset in f, Endothelial cell mPGE synthase-1 immunoreactivity within the myometrial compartment. Scale bars: e and inset, 100 μm; f, 500μm

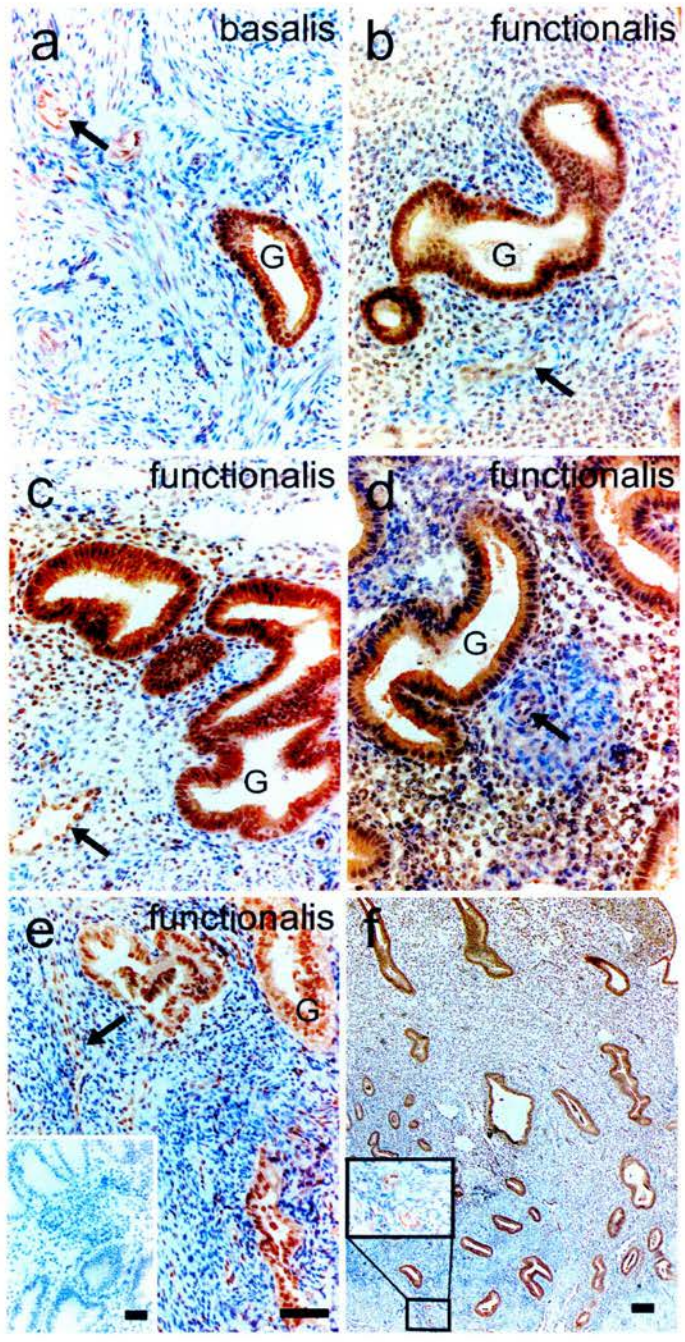

Figure 3.2 Immunohistochemical localization of PGE$_2$ in the basalis and functionalis regions of the human endometrium. Lower PGE$_2$ immunoreactivity was detected in the stromal compartment of the basalis than in the functionalis region (a and b are basalis and functionalis regions, respectively, of endometrial tissue collected during the mid-proliferative phase). In the functionalis region, PGE$_2$ immunoreactivity was detected at all stages of the menstrual cycle and was localized to glandular epithelial (G), stromal and endothelial (denoted by arrows) cells (c, late proliferative; d, early secretory; e, late secretory). Inset in e, Section that was stained with preadsorbed PGE$_2$ during the late proliferative phase (negative control), demonstrating spatial changes in PGE$_2$ immunoreactivity between basalis and functionalis regions of the endometrium and the myometrium. Inset in f, Endothelial cell PGE$_2$ immunoreactivity within the myometrial compartment. Scale bars: e and inset, 100 μm; f, 500 μm

3.3.2 Expression of EP2 and EP4 Receptors in Endometrial Tissue

The expression of two PGE_2 receptors, namely EP2 and EP4 were quantified by quantitative RT PCR analysis. Using immunohistochemistry, the receptors were localised and the possible sites of action of PGE_2 within the endometrium identified. Analysis by quantitative RT PCR demonstrated that the EP4 receptor mRNA (see figure 3.3B) was significantly higher in the biopsies from the late proliferative phase (0.162+/- 0.034; n=5) of the menstrual cycle compared with the early, mid and late secretory phase samples (0.075 ± 0.024, 0.063 ± 0.014 and 0.07 ± 0.033; respectively, $P<0.05$). Whilst the EP2 receptor mRNA displayed a trend of being elevated in biopsies from the mid to late secretory phase (see figure 3.3A), this effect was not significant. It is however, important to take into account that the biopsies are of a heterogeneous cell population. If the individual cell populations were separated, EP2 receptor expression may show a significant change in expression across the menstrual cycle for a specific cell type.

Immunohistochemistry studies localised EP2 receptor expression in the glandular epithelial cells (G), endothelial cells (arrowed) and stromal cells of the endometrium across the menstrual cycle (see figure 3.4). EP4 receptor expression was also demonstrated in the glandular epithelial cells, endothelial and stromal cells (see figure 3.5). However, staining for EP4 was weak across the menstrual cycle except in the late proliferative phase when the immunoreactivity was stronger. EP2 and EP4 receptor expression was shown to be present in both the functionalis and basalis

regions of the endometrium. Preadsorbing with IgG abolished immunoreactivity. (Inset 3.4 A and 3.5C)

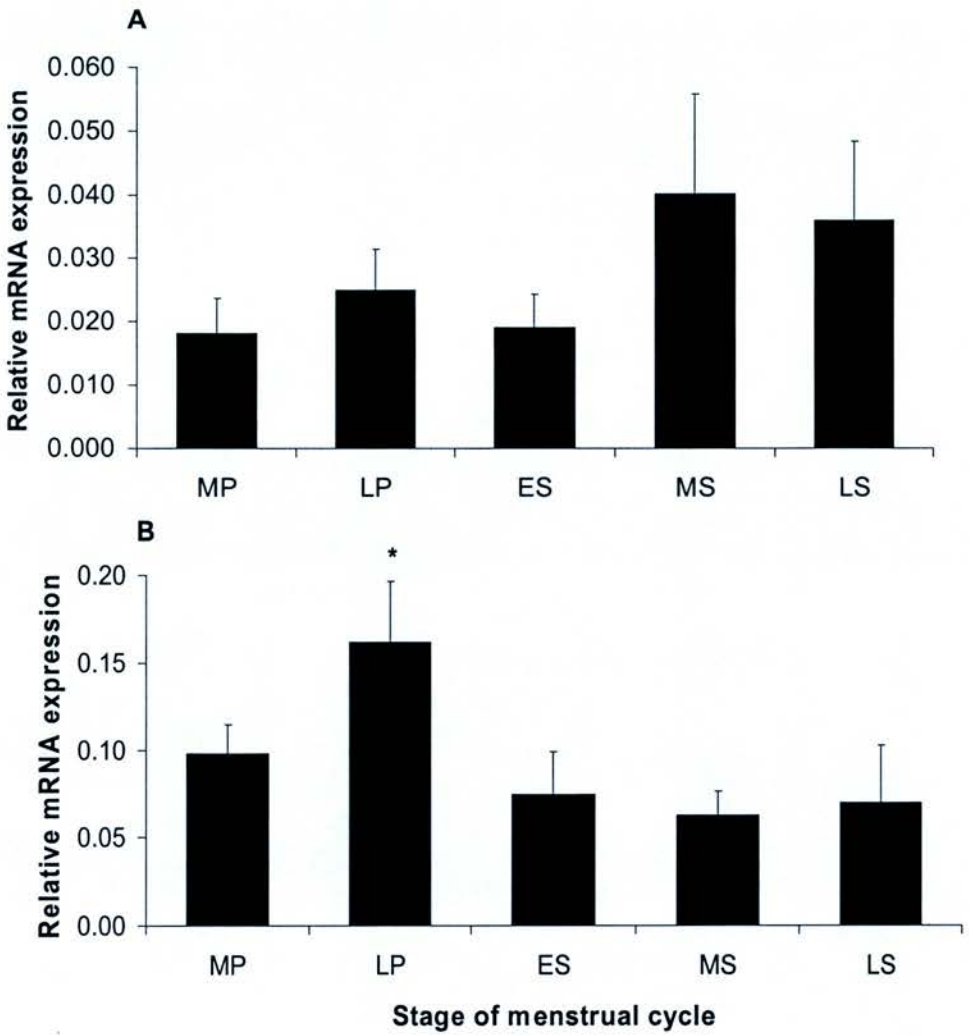

Figure 3.3 Quantitative RT PCR demonstrating relative expression of EP2 (A) and EP4 (B) receptors in midproliferative (MP; n=9), late proliferative (LP; n=5), early secretory (ES; n=7), midsecretory (MS; n=6) and late secretory (LS; n=6) endometrial biopsies. The data are presented as mean ± SEM. * denotes statistical significance relative to other stages of the menstrual cycle ($P < 0.05$)

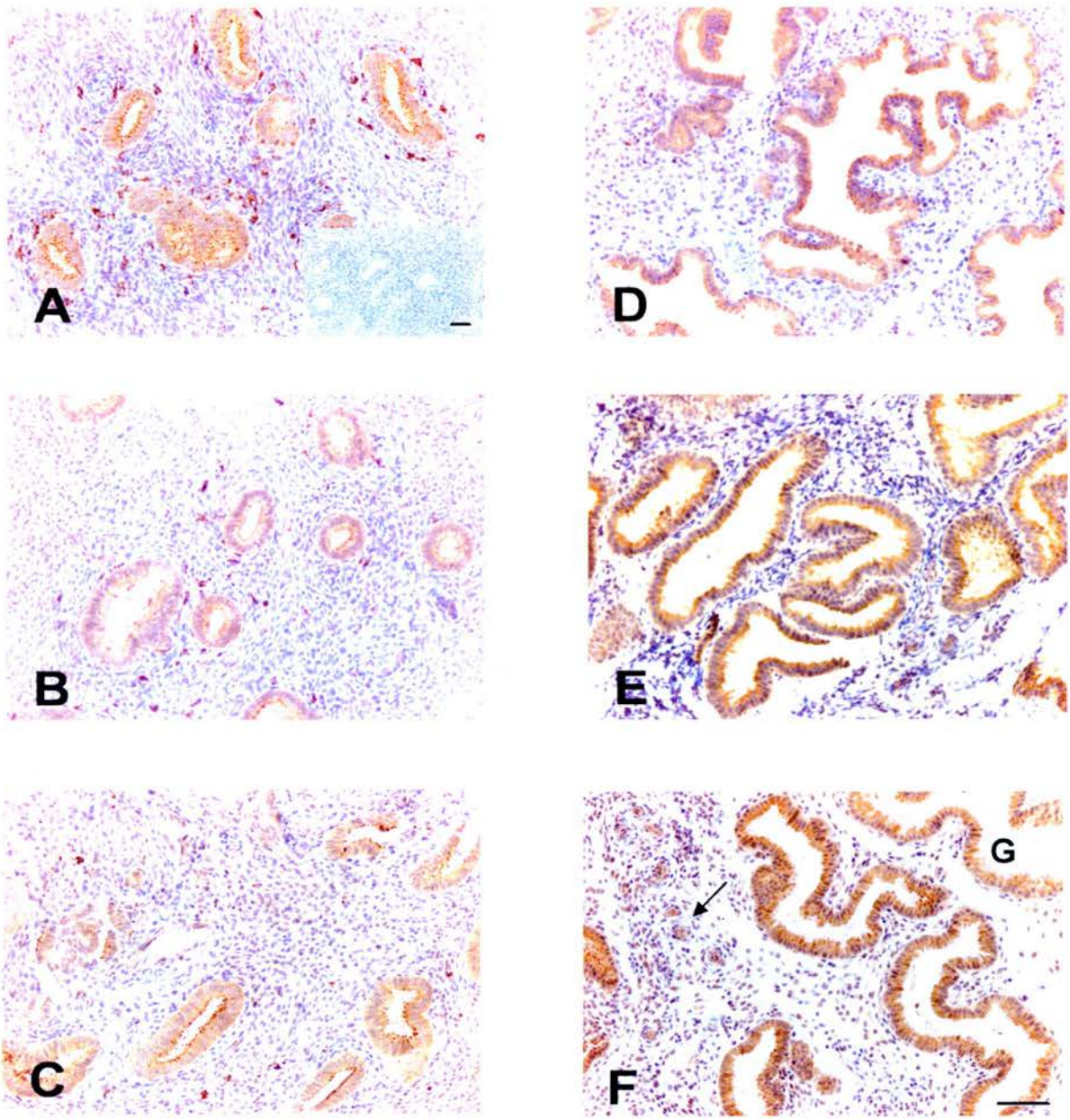

Figure 3.4. Immunohistochemical localization of EP2 receptor detected in human endometrial tissue. EP2 expression is detected in glandular epithelial cells denoted by G, endothelial denoted by arrow and stromal cells during the proliferative phase (A-C) and Secretory phase (D-F) of the menstrual cycle. Inset in A is a negative control (Rabbit IgG). Scale bar is 100 μm.

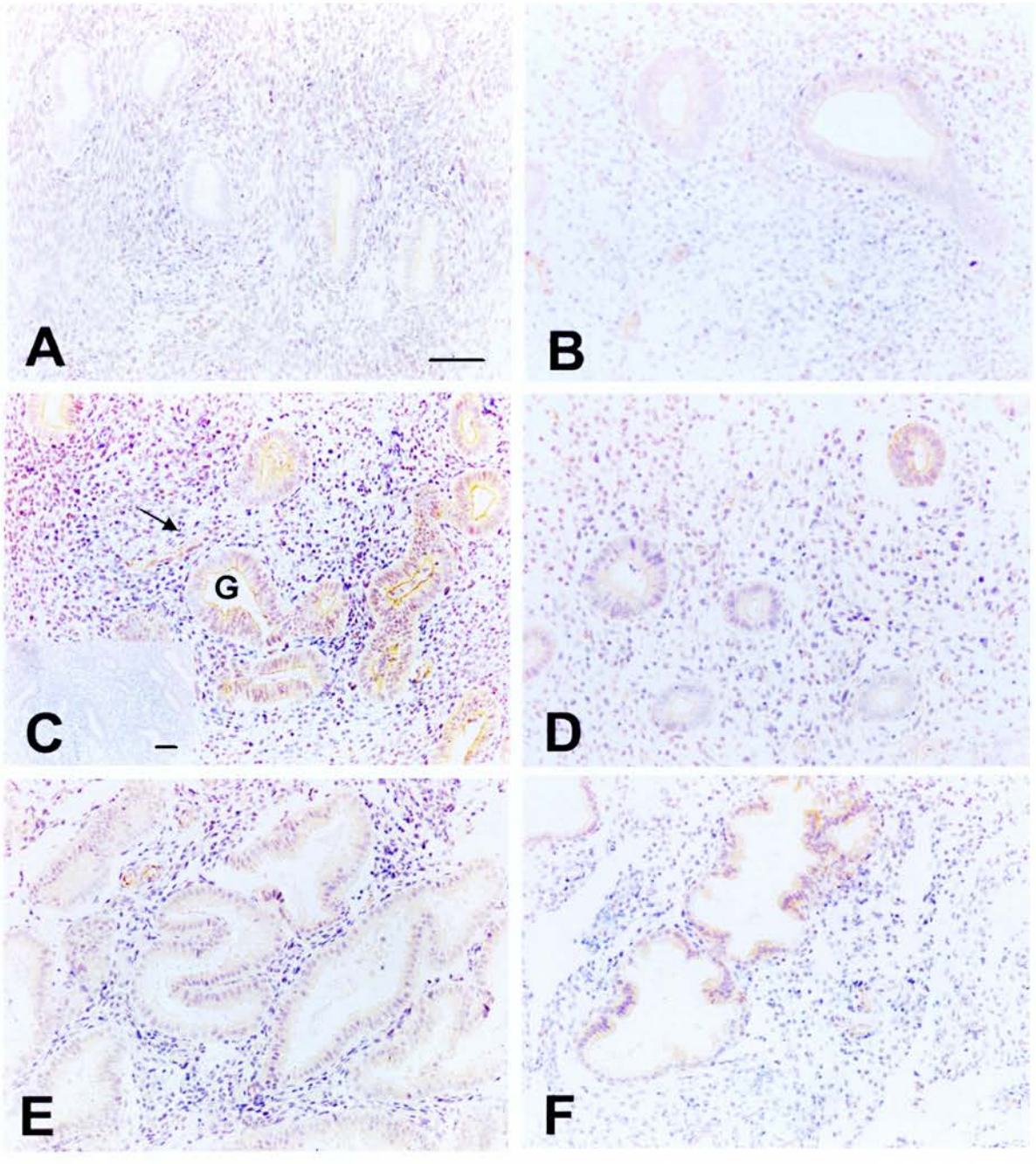

Figure 3.5. Immunohistochemical localization of EP4 receptor detected in human endometrial tissue. (A-C, Proliferative phase and D-F, Secretory phase) EP4 expression is detected in glandular epithelial cells denoted by G, endothelial denoted by arrow and stromal cells during the late proliferative phase of the menstrual cycle (C). Only very weak immunoreactivity was detected in other phases of the menstrual cycle. Inset in C is a negative control (IgG). Scale bar is 100 μm.

3.3.3 Functional Signalling of EP2/EP4 Receptors in Endometrial Tissue

Activation of EP2 and EP4 receptors by PGE_2 results in the generation of cAMP (149). To investigate the signalling functionality of the receptors in the endometrium, cAMP accumulation in response to exogenous PGE_2 was measured by ELISA (see figure 3.6). cAMP generation was significantly higher ($P<0.05$) in biopsies from the proliferative phase of the cycle as compared with the mid and late secretory tissue (3.77 ± 0.85, 1.96 ± 0.28, 1.38 ± 0.23 respectively).

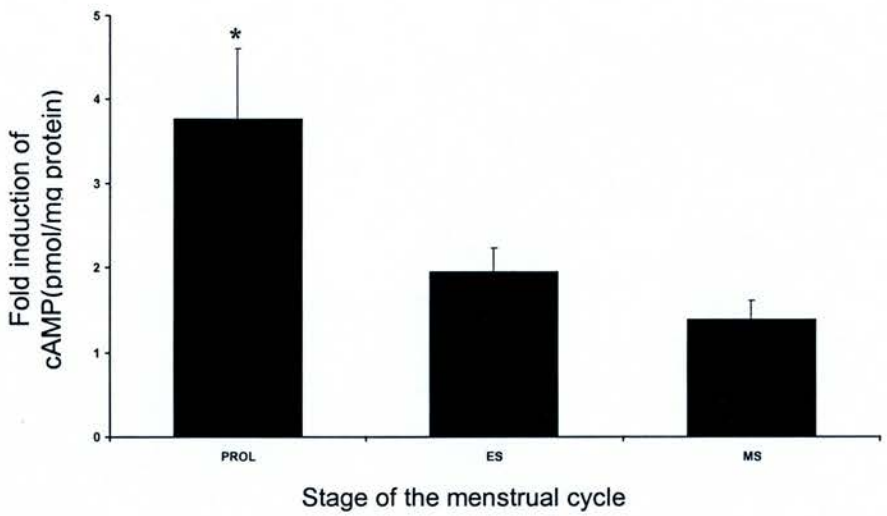

Figure 3.6 Fold induction of cAMP production in endometrial biopsies collected from proliferative (PROL; n=6) early secretory (ES; n=6) and midsecretory (MS; n=6) stages of the menstrual cycle after stimulation with 300nM PGE_2. The data are presented as mean ± SEM. * denotes statistical significance relative to other stages of the menstrual cycle ($P < 0.05$)

3.4 Discussion

This study has demonstrated and localised the expression of mPGES-1 and its product PGE_2 by immunohistochemistry to the stromal, epithelial and endothelial cells of the human endometrium. During the course of my PhD, further forms of PGES have been identified and antibodies have recently become available. Further studies to localise the sites of expression of other PGES isoforms would be interesting to compare their expression. mPGES-1 expression and PGE_2 synthesis was demonstrated across the menstrual cycle with reduced staining in the late secretory phase of the menstrual cycle. This is in agreement with previous in-vitro studies which have confirmed endometrial epithelial and stromal cells can synthesise PGE_2 (243). Furthermore, a reduced PGE_2 biosynthetic capacity has been observed during the late secretory phase (244). PGE_2 is synthesised in cells by the action of the COX enzymes (73). The COX enzymes have previously been localised in the endometrium to epithelial and perivascular cells (4, 23, 102). To investigate the possible sites of action of PGE_2, expression and localisation of two of its G-protein coupled receptors, namely EP2 and EP4 were investigated by immunohistochemistry and quantitative RT PCR. EP2 and EP4 receptor expression was localised to epithelial, endothelial and stromal cells of the endometrium. This observation is confirmed by previous studies localising EP2 receptor mRNA and EP4 receptor mRNA to the epithelial and vascular cells of the endometrium using in situ hybridisation (245). Recent studies have demonstrated other prostanoid receptors to be temporally expressed in the endometrium. FP receptor expression has been reported to be maximal during the mid to late proliferative phase of the menstrual

cycle and predominantly expressed in the glandular epithelial cells (183). Whilst, maximal IP receptor expression is seen during the menstrual phase of the cycle and is localised to glandular epithelial, stromal and endothelial cells (189). The expression of PGE_2 receptors on multiple cell types within the endometrium suggests PGE_2 acts in an autocrine / paracrine manner. This observation further supports the suggested autocrine / paracrine action of PGE_2 previously suggested in the female reproductive tract (65).

EP2 receptor and EP4 receptor expression and functionality were investigated by RT PCR and quantified cAMP generation in response to exogenous PGE_2 respectively. Expression of the EP4 receptor was demonstrated to be significantly elevated in the proliferative phase of the menstrual cycle. cAMP accumulation was also significantly higher in tissues from the proliferative phase compared with those from the secretory phase of the menstrual cycle. The EP4 receptor is associated with Gs coupled signalling and results in enhanced cAMP generation (149). This suggests the enhanced cAMP generated in response to PGE_2 in the proliferative phase is due to the elevated EP4 receptor expression.

The exact role of COX and its products within the endometrium remains to be elucidated. However, numerous studies have implicated COX and its products including PGE_2 in the inhibition of apoptosis, mitogenesis and angiogenesis (65, 137, 138, 211). In vitro studies in Ishikawa, cells, an endometrial epithelial cell line, have demonstrated both PGE_2 and $PGF_{2\alpha}$ enhance cellular proliferation via diverse signalling pathways (183, 234). Overexpression of COX-2 in a rat intestinal

epithelial cell line is associated with elevated PGE_2 which is accompanied with enhanced proliferation and an inhibition of apoptosis via upregulation of antiapoptotic genes (137, 138). A similar mechanism may regulate proliferation of cells in the reproductive tract. The endometrium of menstruating women undergoes a 28 day cycle. During the cycle, the functionalis layer is shed followed by re-growth, resulting in cellular proliferation and angiogenesis during the proliferative phase of the menstrual cycle. Angiogenesis continues during the secretory phase culminating with menstruation when endometrial cells undergo apoptosis. Apoptosis in the glandular epithelial cells of the endometrium has been demonstrated to be very low in the proliferative phase and maximal in the late secretory and menstrual phases of the cycle (246). Elevated levels of the anti apoptotic gene bcl-2 have been reported in the proliferative phase of the menstrual cycle compared with the secretory phase and localised to glandular epithelial cells (36, 247, 248). These studies suggest PGE_2 may play a role in the control of apoptosis within the endometrium.

Overexpression of COX-2 in epithelial cells accompanied by elevated PGE_2 levels increases angiogenic factor expression (138, 207). Once synthesised, the angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) act in a paracrine manner to promote microvascular tube formation and endothelial cell migration (138). Within the endometrium, VEGF has been localised to glandular epithelial cells across the menstrual cycle (249, 250). Furthermore, overexpression of EP2 receptor in Ishikawa cells has recently been demonstrated to induce VEGF expression and secretion (168). The data reported in these recent studies suggest that PGE_2 within the endometrium may play a role in regulating epithelial cell proliferation and uterine

angiogenesis; via the upregulation of growth factors such as VEGF, especially in the proliferative phase when EP4 receptor expression is maximal.

The expression of EP2 and EP4 in endothelial cells in the human endometrium as demonstrated by this study suggests PGE_2 may play a direct role in endothelial cell function. Treatment of endothelial cells with a selective COX-2 inhibitor results in decreased microvascular tube formation (251). Co-treatment with PGE_2 partially reverses this observation. This further supports a role for PGE_2 in mediating endothelial cell and vascular function by acting on its EP receptors both on epithelial and endothelial cells. Regulation of vascular function via EP receptors in epithelial cells is indirect. The regulation is potentiated by the PGE_2 – EP receptor mediated regulation of expression of angiogenic genes such as VEGF in these cells (138).

In summary, this study has localised the expression of mPGES-1 and PGE_2 and two of its receptors, namely EP2 and EP4 within the human endometrium on multiple cell types. The EP2 and EP4 receptors were demonstrated to be functional with maximal expression and signalling of the EP4 receptor during the proliferative phase of the menstrual cycle. Whilst the exact role for PGE_2 remains to be elucidated, PGE_2 may play a role in inhibiting apoptosis, mediating cellular proliferation and angiogenesis within the human endometrium via an autocrine / paracrine action .

Chapter 4

Establishment of endometrial epithelial cells stably transfected with COX-2 cDNA in the sense and antisense directions and the identification of differentially expressed genes

4.1 Introduction

The COX-2 8.3 kb gene is located on chromosome 1, and is an immediate early-response gene that gives rise to a gene product of 4.5kb. COX-2 is rapidly induced following stimulation of quiescent cells by growth factors, oncogenes, and phorbal esters (94, 231, 252-254). COX-2 has a molecular weight of 72kDa and is a glycosylated integral membrane protein located in the endoplasmic reticulum and the nuclear membrane (76). Numerous studies have demonstrated that overexpression of COX-2 in epithelial cells is associated with enhanced production of angiogenic factors (138, 207, 209, 255). These factors act in a paracrine manner to promote endothelial cell migration and microvascular tube formation (138). Furthermore, in vivo and in vitro models have directly demonstrated COX-2 products including PGE_2, $PGF_{2\alpha}$ and PGI_2 induce VEGF expression (207, 209, 210, 256). In the female reproductive tract a role for COX enzymes and its prostaglandin products in normal and pathological angiogenesis, epithelial and endothelial cell fucntion has been proposed (4, 23, 61, 62, 102, 183, 187, 189, 245, 257). In the human endometrium expression/synthesis and signalling of COX-2, PGE_2, and EP receptors co-localise in glandular epithelial and endothelial cells of the normal and neoplastic endometrium (4, 23, 61, 62, 102, 183, 187, 245, 257). Moreover, overexpression of COX enzymes in epithelial cells of the reproductive tract has been shown to promote the expression of various angiogenic factors (65).

Excessive blood loss during menstruation (menorrhagia), defined as > 80ml blood lost per menstrual cycle, with no observed uterine pathology affects 10% of women

of child bearing age. Dysmenorrhoea; which is often associated with painful menstruation and endometriosis; where uterine tissue is found within the pelvic pertitoneum and other extra-uterine sites, are common benign uterine pathologies. These gynaecological complaints are considerable reproductive health problems for women and also place a financial burden on healthcare (43).

A role for COX enzymes has been proposed in benign and neoplastic pathologies of the endometrium such as endometriosis, dysmenorrhoea, heavy menses and endometrial carcinoma (2, 5, 6, 54, 55, 61, 258, 259). Furthermore, several studies have associated heavy menses with abnormalities in prostanoid production such PGE_2 from the uterus (2, 54, 258). PGE_2 synthesis and PGE_2 binding sites in uterine tissues are greater in women diagnosed with heavy menses compared to women with normal blood loss (2-4, 260, 261). Elevated levels of PGI_2 have been shown in menstrual blood collected from women with menorrhagia (54, 262, 263). Furthermore, a shift in the balance of prostanoids synthesised in women with heavy blood loss has been suggested (2, 263). Menorrhagia has been associated with increased synthesis of PGI_2 relative to thromboxane A_2 and elevated PGE_2 relative to $PGF_{2\alpha}$ The elevated prostanoids detected in menstrual flow of patients with heavy menses has lead to the administration of COX enzyme inhibitors as a means of therapy (264). COX enzyme inhibitors such as ibuprofen have been shown to reduce menstrual blood loss (263). This suggests that the degree or duration of menstrual bleeding in women diagnosed with menorrhagia is augmented following elevation of vasodilatatory factors by COX enzyme products. Interestingly, COX-2 has been shown to preferentially metabolise arachidonic acid to PGE_2 and PGI_2 (86).

An elevated level of PGE_2 and $PGF_{2\alpha}$ in the menstrual fluid of women with dysmenorrhoea has been demonstrated compared with women with pain free menstruation (5, 55). A role for COX products in dysmenorrhoea is further implicated by the observation in in-vitro studies where prostanoids released by endometrial explants in response to arachidonic acid is elevated in dysmenorrheic tissue compared with tissue from pain free menstruating women (56). A direct role for prostaglandins in endometriosis remains to be established, but elevated COX-2 expression in endometriotic tissue has been reported (6). Futhermore, the peritoneal fluid of infertile women with endometriosis demonstrates higher levels of prostanoids (7). In endometrial adenocarcinomas, an elevation in COX-2 expression coinciding with enhanced synthesis and secretion of PGE_2, expression and signalling of EP receptors, FP receptors and PPAR has been demonstrated (61, 62, 187).

This study was designed to investigate the potential role of COX-2 in regulating endometrial epithelial cell function. The specific aims of the study were to examine the effect of COX-2 overexpression in an endometrial epithelial cell line (Ishikawa) on PGE_2 and $PGF2_{\alpha}$ secretion, FP and EP receptor expression, and EP receptor signaling. The final aim of this study was to identify genes regulated by COX-2 that may be associated with endometrial function and angiogenesis.

4.2 Materials and Methods

4.2.1 Cell Culture

Ishikawa wild type, COX-2 sense and COX-2 antisense cells were routinely maintained in DMEM nutrient mixture F-12 with Glutamax-1 and pyridoxine, supplemented with 10% fetal bovine serum and 1% antibiotics (500 U/ml penicillin and 500ug/ml streptomycin) at 37°C and 5% CO_2 (v/v). In addition, COX-2 sense and COX-2 antisense cells were maintained in media containing 800µg/ml G418 (Calbiochem, Nottingham, UK).

4.2.2 Construction of Plasmids

The pIRES2 plasmid containing COX-2 cDNA in either the sense or antisense directions was constructed by Dr Kurt Sales, MRC Human Reproductive Sciences Unit, Edinburgh.) Briefly, the pBS(SK-)PSHI vector containing the full length COX-2 gene (kindly supplied by Dr Stephen Prescott, University of Utah, Salt Lake City – UT) was used as the template plasmid. The COX-2 cDNA was excised from the template plasmid and ligated at the EcoR1 site of the pIRES2 vector (Clontech, see Appendix 2). The size of the insert in pIRES2 was confirmed by HINDIII digestion and the orientation of the COX-2 cDNA insert was determined by dideoxy DNA sequencing using sequence specific primers for pIRES2.

4.2.3 Large Scale Plasmid DNA recovery – Maxiprep

Bacterial cultures (100ml) for pIRES2 COX-2 sense and pIRES2 COX-2 antisense were set up overnight in the presence of kanamycin (30$_{\mu}$g/ml). The following day, large scale plasmid recovery was carried out using the endofree™ plasmid maxi kit (Qiagen) according to the manufacturers instructions. Briefly, the bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C. The pelletted cells were resuspended in 10ml buffer P1 (Tris-HCl; 50mM pH 8.0, EDTA; 10mM and RNase A; 100µg/ml). The cell pellet was resuspended by repeated pipetting up and down. To lyse the cells, 10ml buffer P2 (NaOH; 200mM and SDS; 1%) was added and the cells were incubated at room temperature for 5 minutes. To neutralize the cell lysate, 10ml buffer P3 (potassium acetate; 3M, pH 5.5) was added. The lysate was then transferred to a QIA filter, incubated for 10 minutes at room temperature and then filtered. Following this, 2.5ml of buffer ER (Qiagen) was added and the filtered cell lysate incubated on ice for a further 30 min. The filtered lysate was then applied to a pre-equilibrated QIAGEN- tip™. The filtered lysate was then washed twice with 30ml buffer QC (NaCl; 1mM, MOPS; 50mM pH 7.0, and isopropanol; 15%) and the DNA eluted with buffer QN (NaCl; 1.6mM, MOPS 50mM pH7.0 and isopropanol; 15%). The eluted DNA was precipitated by adding 10.5ml isopropanol and then immediately centrifuged at 15 000 x g for 30 min at 4°C. The supernatant was removed and the remaining DNA pellet was washed with 15 ml ethanol (70%) and then centrifuged at 15 000 x g for 15 min at 4°C. Finally, the DNA pellet was air dried and redissolved in 200µl buffer TE (Tris-HCl; 10mM pH 8.0 and EDTA;

1mM). The DNA concentration was then determined by spectrophotometry as described in section 2.5.2.

4.2.4 Transfection of Ishikawa Cells and Selection of Clones

4.2.4.1 Preliminary Transfection Studies

Initial studies were performed in HES cells (kind gift from Dr Douglas Kniss, Ohio State University, Columbia); an endometrial epithelial cell line, to establish a COX-2 overexpressing cell line using the Clontech Tet-Off™ system. Using the Tet-Off system, once transfected, the cells are maintained in cell culture medium containing either tetracycline or a tetracycline derivative. The Tet-Off system allows for a transfected gene of interest to be transcribed only when tetracycline or doxycycline (DOX); a tetracycline derivative, is removed from the cell culture medium. The first attempt to establish a COX-2 Tet-Off system successfully generated HES cell clones transfected with the COX-2 cDNA in the sense direction. However, when screening these clones, COX-2 protein expression was not upregulated in the absence of DOX. Hygromycin (200μM) and G418 (800μg/ml) were used to select cells containing the pTet-Off and pTRE2 (containing the COX-2 cDNA in the sense direction), vectors. The concentrations of hygromycin and G418 were reduced to 100μM and 600μg/ml respectively in an attempt to prevent the successfully transfected cells from dying. However, six successive efforts to establish a HES COX-2 Tet-Off system were unsuccessful as all the cells died within 4 weeks of transfection and during the selection process.

As establishing a HES COX-2 Tet-Off model proved unsuccessful, the pIRES2 vector containing COX-2 in either the sense or antisense directions was then used to set up a stable COX-2 overexpressing system in HES cells (method as described in section 4.2.4.2). During the selection process with G418, a large number of COX-2 sense and COX-2 antisense colonies grew. 25 colonies were picked in the COX-2 sense direction and 25 colonies in the COX-2 antisense direction were picked. COX-2 protein expression by the colonies was determined by Western blot analysis. Two COX-2 sense clones demonstrated overexpression of COX-2 and one COX-2 antisense clone displayed a reduced but detectable level of COX-2 protein. To confirm functionality of the transfected COX-2 cDNA, PGE_2 and $PGF_{2\alpha}$ synthesis was measured by ELISA as described in section 4.2.7. PGE_2 secretion was significantly increased ($P<0.05$) in the culture media of both HES COX-2 sense clones S7 and S17 (2559.88 ± 533.50 pg/ml and 837.17 ± 29.58 pg/ml respectively) relative to the HES COX-2 AS clones (675.07 ± 238.22 pg/ml). Co-culture of the cells with the COX-2 specific inhibitor NS398 significantly reduced ($P<0.05$) the PGE_2 generation in the COX-2 sense clones S7 and S17 relative to those treated with AA alone (389.68 ± 155.94 pg/ml and 150.09 ± 35.14 pg/ml respectively) Interestingly, $PGF_{2\alpha}$ was only significantly elevated ($P<0.05$) in one HES COX-2 sense clone, S7, relative to the HES COX-2 antisense clone (489.85 ± 42.39 pg/ml and 182.30 ± 36.33 pg/ml respectively). Co-culture of the S7 COX-2 sense cells with NS398 significantly reduced ($P<0.05$) $PGF_{2\alpha}$ secretion in the culture medium relative to COX-2 sense cells treated with AA alone (173.38 ± 33.09 pg/ml).

Having established a COX-2 overexpressing model in the HES cells, a number of experiments were performed to investigate differential expression of growth factors and altered proliferative rates. VEGF, bFGF, Ang-1, Ang-2 and PGES protein expression were all undetectable in HES WT, COX-2 sense and COX-2 antisense cells as measured by Western Blot. An initial proliferation studies using BrdU incorporation performed according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany) showed no difference between the COX-2 sense and the COX-2 antisense cells and these studies were not pursued further.

Since only one HES COX-2 sense clone displayed significantly elevated $PGF_{2\alpha}$ secretion and the HES COX-2 antisense clone expressed detectable COX-2 protein, it was decided to investigate establishing a COX-2 overexpressing model in a different cell line. Performing a transient transfection in Ishikawa cells; an endometrial epithelial cell line, demonstrated a marked increase in COX-2 protein expression in the COX-2 sense cells relative to Ishikawa wild type and an undetectable level of COX-2 protein in Ishikawa COX-2 antisense cells. Ishikawa cells were then transfected with the pIRES2 vector containing COX-2 in either the sense or antisense directions as described in section 4.2.4.2 and all further studies in this thesis were performed using this model.

4.2.4.2 Ishikawa Studies

Ishikawa cells (European Collection of cell culture, Centre for applied Microbiology, Wiltshire, UK) were transfected with the pIRES2 vector containing COX-2 cDNA

(1μg) in either the sense or antisense directions as follows. Ishikawa cells were seeded at a density of 120 000 in 12 well plates in complete media (1ml per well). The cells were allowed to attach overnight at 37°C in a humidified 5% CO_2 incubator. The following day, the Ishikawa cells were transfected (6 wells in the COX-2 sense direction and COX-2 antisense direction respectively) using pfx-5 (Invitrogen) in a ratio of 9:1 pfx-5:cDNA diluted in optimem (Gibco). Control cells were also incubated in optimem alone and optimem containing pfx-5. Transfection efficiency was assessed by transfecting ishikawa cells with pcDNA6/V5/His/LacZ (Invitrogen). Transfected and control cells were incubated for 4 hours at 37°C in a humidified 5% CO_2 incubator. The media was aspirated and replaced with complete media. Cells were allowed to grow for 48 hours. The transfected cells were then seeded in large flasks together with Ishikawa wild type cells in a ratio of 1:10. To select the successfully transfected cells, the cells were incubated in complete media containing G418 (800μg/ml). In order to establish when all untransfected cells had died, one flask was seeded as a control with Ishikawa wild type cells in complete media in the presence of G418 (800μg/ml). The media was aspirated every two to three days on all cells and replaced with fresh complete media containing G418 (800μg/ml). This routine was maintained until the untransfected cells had died and the G418 selected clones had grown to an appropriate size to be picked, whilst remaining separate colonies. The clones were picked using cloning cylinders (Sigma). Briefly, the cylinders were placed around a colony, the cells were trypsonised and the individual colonies transferred to separate wells on a 24 well plate. A total of 120 clones with COX-2 cDNA in the sense direction and 60 clones with the COX-2 in the antisense directions were picked. The clones were maintained

in 6 well plates as described in section 2.3 and 4.2.1. All the clones were screened for COX-2 protein expression by Western blot analysis. Based on the COX-2 protein expression, four clones containing COX-2 cDNA in the sense direction and 2 clones containing COX-2 cDNA in the antisense directions namely S22, S26, S47 and S72; AS15 and AS47 respectively were chosen. These clones were used to measure PGE_2 and $PGF_{2\alpha}$ secretion. Following this study, all further investigations were performed using the COX-2 sense 72 clone and the COX-2 antisense 15 clone as these generated the highest and lowest levels of PGE_2 and $PGF_{2\alpha}$ respectively. Initial BrdU incorporation and apoptosis ELISA studies (Roche Diagnostics GmbH, Mannheim, Germany) indicated there were no differences in proliferation or apoptosis between the COX-2 sense cells and the COX-2 antisense cells and these studies were not pursued further.

4.2.5 β-Galactosidase Assay

Transfection efficiency was assessed using β-Galactosidase (X-gal) with the Ishikawa cells tranfected with the pcDNA6/V5/His/LacZ (Invitrogen) cDNA grown for 48 hours. The media was aspirated and the cells were then washed three times with phosphate buffered saline (PBS) and then fixed with 0.2% glutaraldehyde / 2% formaldehyde in PBS for 5 min. The fixing solution was then aspirated and the cells washed three times in PBS. Finally, the cells were incubated overnight at 37°C in the presence of the staining solution ($K_3Fe(CN)_6$; 5mM, $K_4Fe(CN)_6$; 5mM, $MgCl_2$; 2mM, X-gal; 1mg/ml). The following day, those cells successfully transfected were

stained blue. The transfection efficiency was calculated by counting the number of blue cells and expressed as a percentage of the total cells.

4.2.6 Protein Extraction and Western Blot Analysis

Protein extraction, quantification and Western blot analysis were performed as described in section 2.4. Briefly, Ishikawa wild type, COX-2 sense and COX-2 antisense cells were seeded at a density of $7.5x10^5$ cells in six well plates, allowed to attach for 24 hours in complete media and then cultured in serum free media overnight to reach the desired confluency of 70% (n=4 independent experiments). A total of 20μg protein for cathepsin D expression and 40μg protein for COX-2, COX-1 and β-actin, were denatured and subjected to SDS-PAGE on 4-12% Tris-glycine gels (Invitrogen, Paisley, UK). The proteins were transferred onto PVDF membrane and blocked for 1 hour in TBS-Tween (50mM Tris-HCl, 150mM NaCl and 0.05% (v/v) Tween 20 containing 5% skimmed milk powder). The membranes were probed with one of the following antibodies: COX-2 (sc-1745; at dilution of 1:1000), COX-1 (sc-1752; at dilution of 1:500), cathepsin D (sc-6486; at dilution of 1:2000), β-actin (sc-1616; at dilution of 1:1000) or Plasminogen (sc-15034; at dilution of 1:500) overnight followed by rabbit antigoat conjugated to alkaline phosphatase secondary antibody at a dilution of 1:30 000 (Sigma, Poole, UK). All the primary antibodies were purchased from Santa Cruz Biotechnology (Autogenbioclear, Whiltshire, UK). The membranes were developed and revealed by phosphorimager analysis using the ECF chemifluorescence system according to the manufacturer's instructions (Amersham Biosciences UK Ltd, Little Chalfont, UK). The molecular weights of the

proteins were determined by comparing mobility on the gel with a molecular weight standard (Invitrogen, Paisley, UK). Proteins were quantified by densitometry using STORM 860 system (Molecular Dynamics, Amersham Biosciences, Buckinghamshire, UK). Relative expression of cathepsin D protein was calculated by normalising with β-actin and expressed as mean ± SEM.

4.2.7 PGE_2 and $PGF2_\alpha$ assay

Ishikawa wild type, COX-2 sense and COX-2 antisense cells were seeded in 6 well plates at a cell density of 2.5 $X10^5$ cells per well. Cells were left to attach overnight in complete media (2ml). The following day, the complete media was aspirated and the cells washed twice with PBS (2ml), the PBS was aspirated by pipette and replaced with serum free media (2ml) overnight. Arachidonic acid to a final concentration of (5μg/ml) was added to the media the following day ± NS398 (10μM), the COX-2 selective inhibitor, and the cells were incubated for a further 48 hours. The media was then aspirated by pipette and transferred to an eppendorf tube for analysis. For the PGE_2 assay, 1ml of media was removed as described and added to 1ml of methyloximating solution (Methyloxyamine buffer, pH 5.6) to stabilize the PGE_2. The samples were stored at -20°C until further use. PGE_2 and $PGF_{2\alpha}$ secretion in the culture media was assayed using an ELISA as described by Denison et al (265). Initially, 96 well plates (amine-binding plates; Costar, High Wycombe, United Kingdom) were coated with donkey antirabbit antibody and were then coated with rabbit IgG (1mg/ml diluted in PBS with 1% carbonate buffer, pH 9.6) at 200μl/well for 16 hours at 4°C. The solution was aspirated and blocking solution (50mM

glycine,10 mg/ml BSA) was added to the wells (25µl/well) for 2 hours at 23°C. Following washing, donkey antirabbit serum (Scottish Antibody Production Unit, Carluke, United Kingdom) was added to each well before washing and air drying. The plates were stored at 4°C with desiccant. The link was prepared by ether extraction and purified by reverse phase chromatography using 20mg of synthetic PGE_2, 320µl of dry dimethylformamide, 3µl butylchlororomate and 0.05nM biocytin. The link and antisera were diluted $1:1.5X10^6$ in PO_4 0.5M pH8.0 and 1:50 000 in ELISA buffer (150mM NaCl, 100mM Tris-HCl, 0.05% Tween 20, 50mM phenol red, 1 mM 2-methylisothiazolone, 1mM bromonitrodioxane, 2mM EDTA, 2mg/ml BSA to a final pH of 7.2) respectively. For the $PGF_{2\alpha}$ assay, the ELISA buffer contained no Tween. The standard (5120pg/ml) was then serially diluted 9 times in buffer. For the PGE_2 assay, the standards were diluted in ELISA buffer containing 25% MOX and Tween, the $PGF_{2\alpha}$ standards were diluted in ELISA buffer alone. The standards at concentrations of 5120pg/ml, 2560pg/ml, 1280pg/ml, 640pg/ml, 320pg/ml, 160pg/ml, 80pg/ml, 40pg/ml, 20pg/ml and 10pg/ml were used to construct the standard curve. Samples and synthetic standards (100µl) were added in duplicate to the plate followed by 50µl of the diluted link and 50µl of the diluted antisera to all wells except for the non specific binding (NSB) and the maximum binding B_0 control wells. Link (50µl) and ELISA buffer (100µl) alone were added to the NSB wells, for the B0 wells, link (50µl), ELISA buffer (100µl) and antisera (50µl) were added. The plates were incubated overnight at 4°C. The following day, the contents of the wells were aspirated and the plates washed 4 times, once for 30 seconds on an orbital shaker. Following the wash, 100µl /well of streptavidin-peroxidase (0.2 U/ml) was added to each well on the plate. The plates were incubated

for 20 minutes at 23°C on an orbital shaker. Plates were then washed and 200µl/well of the substrate (0.3 g/liter urea-hydrogen peroxide, 0.1g/l tetramethylbenzene in 100 mM sodium acetate, pH 6.0) was added for 10 minutes. The reaction was stopped with 100µl / well sulphuric acid (1 M). The absorbance was measured at 450 nm using a Multiscan® MCC/340 plate reader. Average PGE_2 and $PGF_{2\alpha}$ concentrations of the media samples were determined from the standard curve constructed by extrapolation using the Assay Zap software (Biosoft).

4.2.8 Quantitative RT PCR

Quantitative RT PCR was performed as described in section 2.5.5. Briefly, to quantify the overexpression of COX-2, Ishikawa wild type and COX-2 sense cells were subjected to quantitative RT PCR. The effect of COX-2 on EP and FP receptor expression, was investigated by quantitative RT PCR in Ishikawa wild-type, COX-2 sense clone 72 and COX-2 antisense clone 15 cells. A possible autocrine action of PGE_2 on EP receptor expression was investigated with Ishikawa wild type cells. For all experiments, $5x10^5$ cells were seeded in 6 well plates and allowed to attach for 24 hours in complete media, and then cultured in serum free media overnight. The experiments to quantify the expression of COX-2 and determine the effect of COX-2 on EP and FP receptor expression were performed in the absence of NS398 as recent data has suggested NS398 can upregulate the expression of EP receptors (266). To identify a possible autocrine action of PGE_2 on EP receptor expression, Ishikawa wild type cells were cultured in serum free media containing the non selective COX

inhibitor indomethacin (3μg/ml). Cells were harvested with Tri-Reagent and the RNA isolated and reverse transcribed as described in section 2.5

4.2.9 cAMP Turnover

Functionality of the EP receptors was investigated by measuring cAMP generation in Ishikawa wildtype, COX-2 sense and COX-2 antisense cells. Cells (2×10^5) were seeded in complete media (2ml/well) in 6 well plates. Cells were allowed to grow for 24 hours. The cells were then washed twice with PBS, and then synchronised by incubating overnight with serum free medium. The following morning, the culture medium was aspirated and replaced with serum-free medium containing IBMX (Sigma) to a final concentration of 1 mM for 1.5 hours at 37°C. Cells were then stimulated with 0 or 100 nM PGE_2 for 10 minutes. Following stimulation, the medium was removed and the cells lysed in HCl (0.1 M). cAMP concentration was quantified by ELISA using a cAMP kit (Biomol) as described in section 2.6 and normalised to protein concentration of the lysate. Protein concentrations were determined using a protein assay kit as described in section 2.4.2 The data are presented as mean (± SEM), fold induction of cAMP in COX-2 sense and antisense cells after treatment with PGE_2 where fold induction was calculated relative to the wild-type samples. The intra- and interassay coefficients of variation were 8.3% and 11.6% respectively.

4.2.10 cDNA array Analysis

Differential gene expression in the COX-2 sense and antisense cells was assessed using cDNA array analysis. COX-2 sense and COX-2 antisense cells were grown to approximately 70% confluency in complete media. The media was aspirated by vacumn suction and the cells were washed twice with PBS (5ml) and then cultured in serum free media overnight. Following overnight culture, the media was aspirated by vacumn suction and the cells were then harvested by washing twice with PBS (10ml) followed by 5ml trypsin-EDTA (0.1% trypsin and 0.04% EDTA in PBS). The flasks of cells were then incubated for 5 minutes at 37°C. Subsequently, the cells were resuspended in PBS (10ml) and counted using a heamacytometer. Following this, the cells were pelleted by centrifugation for 5 minutes, 1000 rpm. The supernatant was aspirated by pipette and the cells were snap frozen on dry ice and stored at –70°C. The cell pellets were sent to Clontech Laboratories Inc. (Palo Alto, CA, USA) for custom cDNA array analysis using the Atlas plastic human 8K Microarray service (www.clontech.com).

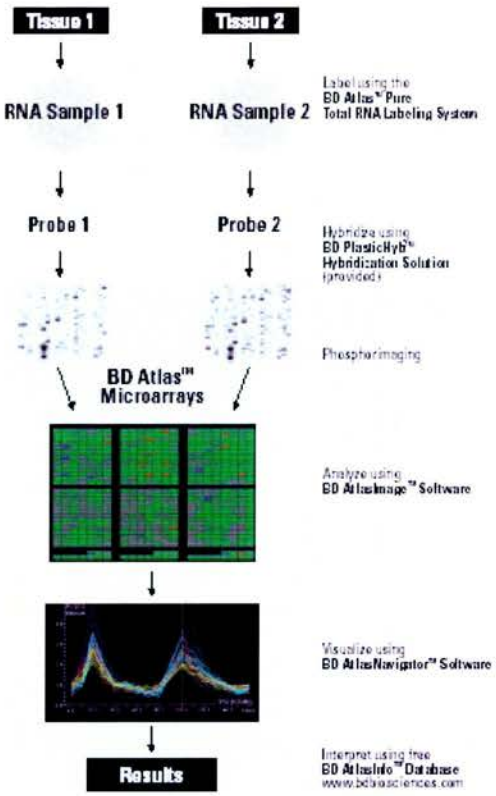

Figure 4.1 Schematic flow diagram for the cDNA array hybridasation procedure (reproduced from the Clontech Plastic Microarray Users Manual)

The procedure for performing the cDNA array was briefly as follows (see figure 4.1). Initially RNA was extracted from the cells of the COX-2 sense and the COX-2 antisense cells. cDNA probes were synthesised, labelled with ^{32}P and then hybridised to the array. The arrays were visualised using a phosphorimager and were analysed using AtlasImage software and expressed as a comparison between the COX-2 sense and COX-2 antisense cells.

4.2.11 Determination of Angiostatin Generation

Ishikawa wild type, COX-2 sense and COX-2 antisense cells were seeded in 6 well plates at a density of $3.5x10^5$, allowed to attach for 24 hours in complete media (2ml). The cell media was aspirated by pipette and the cells were washed twice with PBS (2ml) and then cultured in serum free media (1.5ml) overnight (n=3 independent experiments). Following overnight culture, the media was collected, by pipette, transferred to a centrifuge tube and spun at 1000 rpm for 5 minutes to pellet any cell debris. The supernatant was aspirated by pipette transferred to a new centrifuge tube and stored at -20°C until further use. In order to investigate the differential cleavage of plasminogen to angiostatin by the 3 cell lines, WT, AS and S, the previously frozen media was thawed on ice, aliquots of the thawed cell media (100µl) were pippetted into centrifuge tubes and incubated with 25µg /ml plasminogen at 37°C in a water bath for 0, 4, 8 or 24 hours. In order to investigate whether cathepsin D mediates the cleavage of plasminogen to angiostatin, 100µl of media from COX-2 antisense cells were incubated in a centrifuge tube in the presence or absence of 1µM pepstatin A, a cathepsin D inhibitor (Sigma, Poole, UK) for 8 hours at 37°C in a water bath. Subsequently, a total of 20µl of each reaction was denatured and subjected to SDS-PAGE on 4-12% Tris-glycine gels (Invitrogen, Paisley, UK) as described in section 2.4.3. The generation of angiostatin from plasminogen was assessed by Western blot analysis and probed with a plasminogen antibody as described in sections 2.4.4 and 4.2.6

4.3 Results

4.3.1 COX-2 and COX-1 Protein Expression in Wild Type and Transfected Cells

Stable transfection of the Ishikawa human endometrial epithelial cells with the pIRES2 vector containing COX-2 cDNA in the sense direction resulted in the overexpression of COX-2 protein. Of the COX-2 sense clones picked, Western blot analysis revealed strong COX-2 protein expression detected as 71 kDa band in 24 of the COX-2 sense cell clones (S) compared with the COX-2 antisense (AS) and wild type (WT) cells (see figure 4.2A). Basal levels of COX-2 were detected in the wild type Ishikawa cells that on transfection with COX-2 antisense cDNA was reduced to undetectable levels in 3 of the COX-2 antisense cell clones. (see figure 4.2A). Stable transfection of the COX-2 cDNA in either the sense or antisense direction had no effect on COX-1 protein expression (see figure 4.2B); no difference was detected in COX-1 protein levels between the wild type, COX-2 sense or COX-2 antisense cell lines used for the ongoing studies.

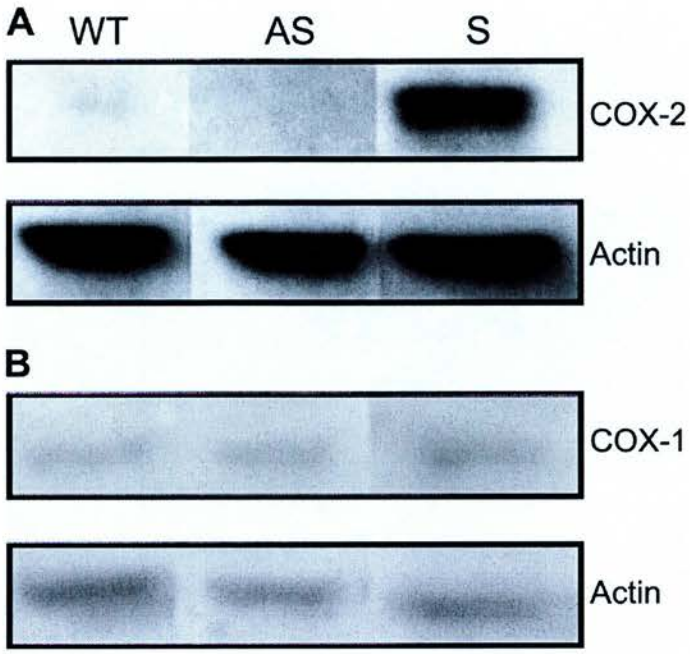

Figure 4.2 Western blot analysis of 40ug protein from wild-type Ishikawa cells (WT) and Ishikawa cells stably transfected with COX-2 cDNA in the sense (S) clone S72 or antisense (AS) clone AS15 directions. A, Specific bands for COX-2 and β-actin proteins were detected at approximately 72 and 46 kDa, respectively. B, COX-1 and β -actin proteins were detected by specific bands at 71 and 46kDa respectively.

4.3.2 Relative COX-2 mRNA Expression in COX-2 Sense Cells and Wild Type Cells

To quantify the COX-2 overexpression, quantitative RT PCR was performed on Ishikawa wild-type and the COX-2 sense 72 clone cells (see figure 4.3). The relative COX-2 mRNA expression was significantly increased ($p<0.05$) in the COX-2 sense 72 clone cells relative to the Ishikawa wild type cells (81.2 ± 8.10 and 9.44 ± 1.94 respectively).

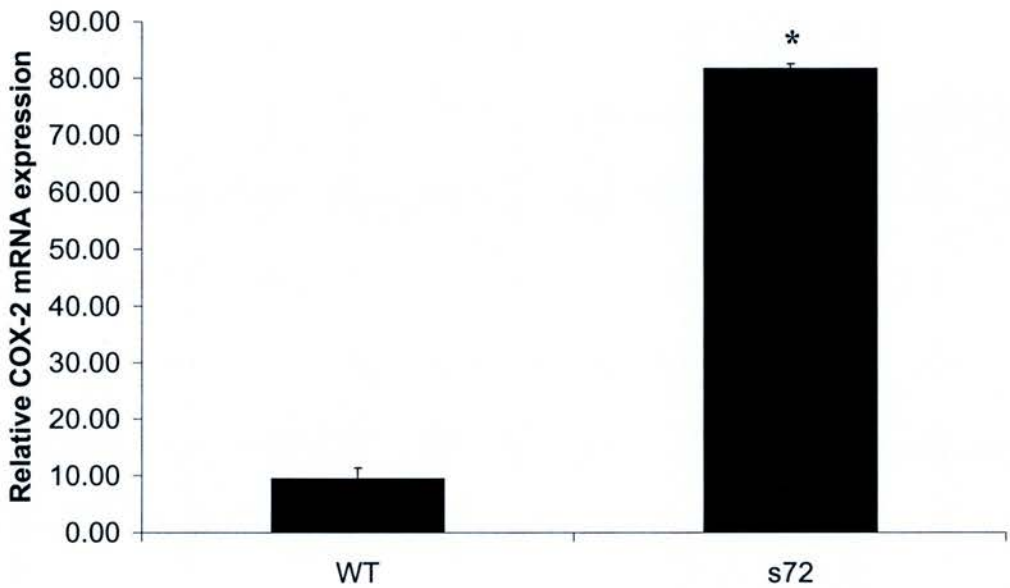

Figure 4.3 Relative expression of COX-2 mRNA in Ishikawa wild type (WT) cells and Ishikawa cells stably transfected with COX-2 in the sense direction (S). The data are mean ±- SEM, n=3. * denotes statistical significance relative to WT ($P < 0.05$)

4.3.3 PGE_2 and $PGF_{2\alpha}$ Secretion

In order to confirm functionality of the transfected cDNA, PGE_2 and $PGF_{2\alpha}$ synthesis were measured by ELISA in four COX-2 sense clones, two COX-2 antisense clones and Ishikawa wild type cells (see figure 4.4). PGE_2 secretion was significantly increased ($P<0.05$) in the culture media of the COX-2 sense clones compared with the COX-2 antisense clones. The COX-2 sense 72 clone generated the highest level of PGE_2 into the culture media (2391.25 ± 311.09 pg/ml) compared with wild type and COX-2 antisense 15 clone which generated the lowest level of PGE_2 (359 ± 71.54 pg/ml and 78.96 ± 50.9 pg/ml respectively). Co-culture of the cells with the specific COX-2 enzyme inhibitor NS398 abolished the increased secretion of PGE_2 in the COX-2 sense cells. Similarly, $PGF_{2\alpha}$ secretion was significantly elevated in the COX-2 sense clones relative to the COX-2 antisense clones, the highest and lowest levels of $PGF_{2\alpha}$ detected were in the COX-2 sense 72 clone and the COX-2 antisense 15 clone respectively. The $PGF_{2\alpha}$ generation by the COX-2 sense 72 clone was significantly increased ($p<0.01$) (1996.73 ± 361.03 pg/ml) relative to the wild type and COX-2 antisense 15 cells (561.30 ± 114.45 pg/ml and 175.80 ± 38.79 pg/ml respectively). The increased secretion of $PGF_{2\alpha}$ was abolished when the cells were co-cultured with the COX-2 selective inhibitor NS398.

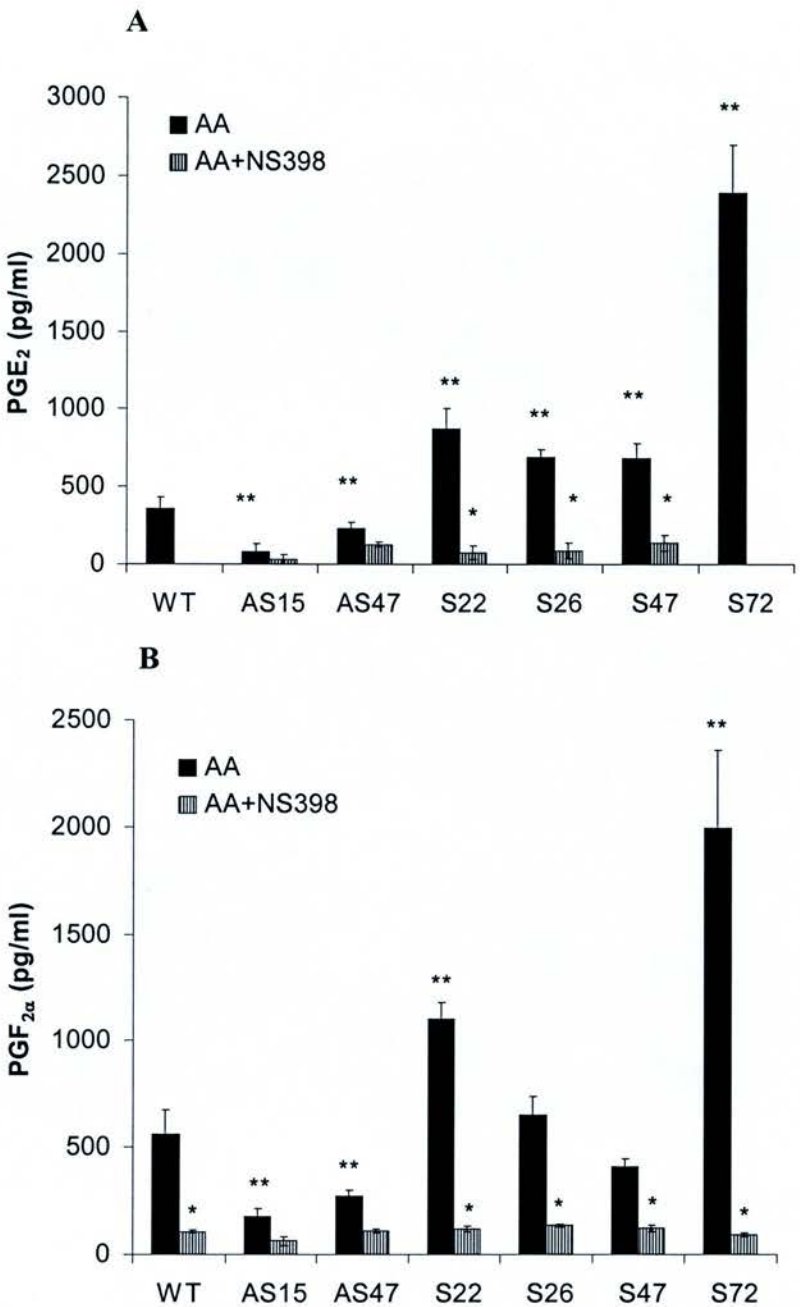

Figure 4.4 The functionality of the transfected COX-2 was assessed by ELISA to measure PGE_2 secretion (A) and $PGF_{2\alpha}$ secretion (B) by wild-type (WT), COX-2 antisense (AS) and COX-2 sense (S) cell clones into culture media following incubation with 5 μg arachidonic acid (AA) for 48h in the presence or absence 10 uM of the COX-2 enzyme inhibitor NS398. The data are mean ± SEM of n = 4 experiments. ** denotes statistical significane relative to AA treated Wild Type, ** denotes statistical significance relative to AA treated in same cell type ($P < 0.05$).

4.3.4 Effect of COX-2 Overexpression on EP and FP Receptor Expression

In order to maximise the effects observed, all further studies were performed using the COX-2 antisense 15 clone and the COX-2 sense 72 clone; which generated the lowest and highest levels of PGE_2 and $PGF_{2\alpha}$ in response to arachidonic acid respectively. To investigate the effect of COX-2 overexpression on EP and FP receptors, real time quantitative RT PCR was performed to quantify the expression of FP, EP1, EP2, EP3 and EP4 receptors (see figure 4.5 A, 4.5 C). Relative expression of EP2 and EP3 mRNA (compared to expression levels detected in wild type cells) was significantly higher ($P<0.05$) in the COX-2 sense cells compared with COX-2 antisense cells (EP2 receptor: 3.73 ± 0.43 vs 1.19 ± 0.39 for COX-2 sense and antisense cells respectively; EP4 receptor: 2.35 ± 0.3 vs 1.03 ± 0.105 for COX-2 sense and antisense cells respectively). EP1 mRNA was not detectable in any of the cell lines and EP4 and FP receptor expression showed no significant differences between the COX-2 sense and antisense cells.

4.3.5 cAMP Generation

Functionality of the EP receptors was investigated by measuring the generation of cAMP in COX-2 sense, COX-2 antisense and wild type cells in response to treatment with 100nM PGE_2 for 10 minutes (see figure 4.5B). Fold induction of cAMP relative to that measured in wild type cells was significantly higher ($P<0.05$) in the COX-2

sense cells compared with the COX-2 antisense cells (2.57 $\pm$ 0.16 vs 0.88 $\pm$ 0.33 for COX-2 sense and antisense cells respectively).

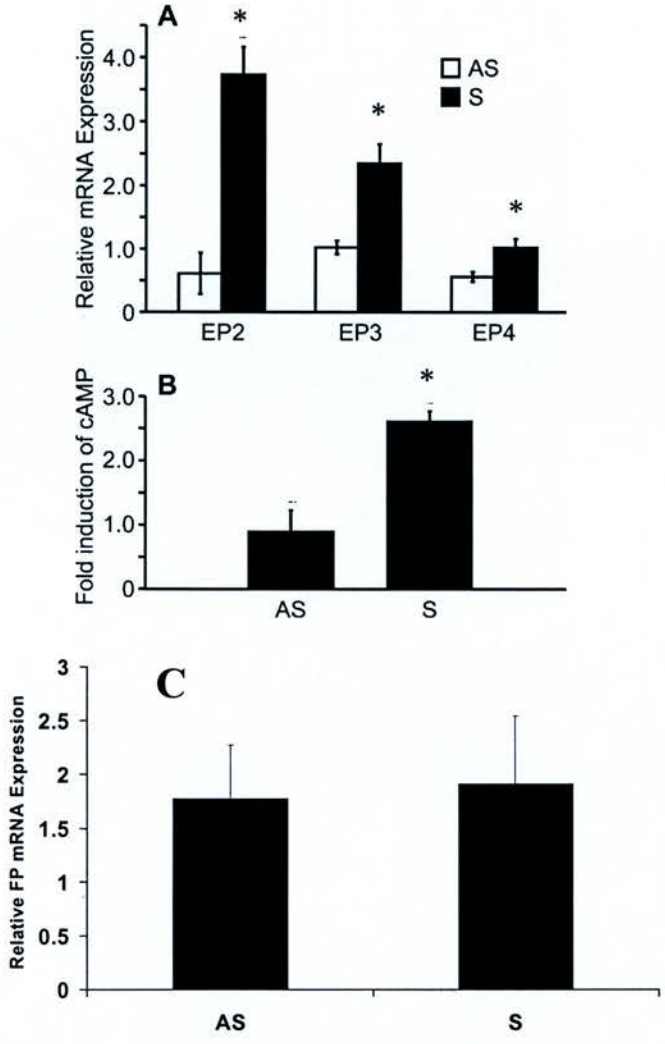

Figure 4.5 (A) Relative expression of EP2, EP3 and EP4 receptors in Ishikawa cells stably overexpressing COX-2 in either the sense (S) or antisense (AS) directions. Relative expression was determined by dividing expression detected in COX-2 sense and antisense cells by expression detected in wild-type cells. The data are mean ± SEM of n=3 experiments. * denotes statistical significance relative to AS ($P< 0.05$). **(B)**, ELISA for cAMP generation. Fold induction of cAMP generation in COX-2 antisense (AS) and COX-2 sense (S) cells following treatment with 100nM PGE_2 for 10 min. Fold induction is calculated by dividing cAMP generation in wild-type cells. The data are mean ± SEM of n = 4 experiments. * denotes statistical significance ($P < 0.05$). **(C)**, Relative expression of FP receptors in COX-2 sense (S) and antisense (AS) cells. Relative expression was determined as described for A. The data are mean ± SEM of n=3 experiments.

4.3.6 Autocrine Action of PGE_2 on EP Receptor Expression

To investigate a possible autocrine action of PGE_2 on its EP receptors in endometrial epithelial cells, we measured EP receptor expression in wild type cells following overnight culture in the presence of the non selective COX inhibitor indomethacin and treatment with exogenous PGE_2 (100nM) for 0,4,6,24 hours (see figure 4.6). Using real time quantitative RT PCR, EP2 mRNA expression was significantly higher (1.56 ± 0.28 $P < 0.05$) following 4 hour treatment of PGE_2 compared with untreated wild type cells. There was no significant difference in EP3 and EP4 RNA expression following treatment with PGE_2. EP1 RNA expression was undetectable both pre and post culture with PGE_2.

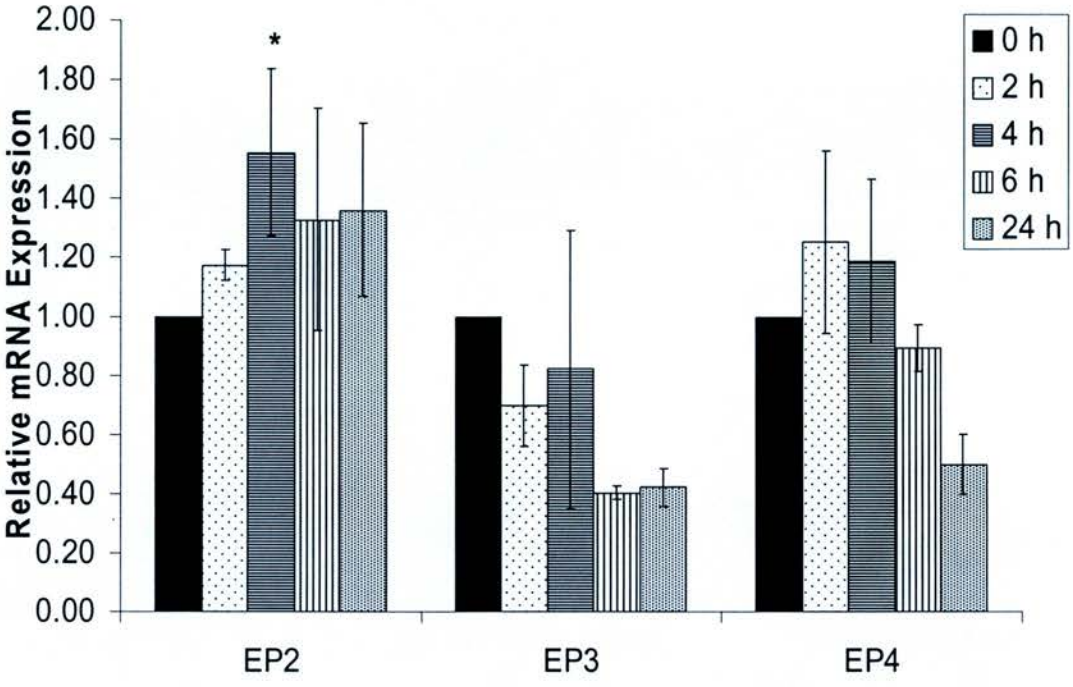

Figure 4.6 Relative expression of EP2, EP3 and EP4 receptors in wild-type Ishikawa cells following overnight culture in the presence of the non selective COX inhibitor indomethacin and stimulation with exogenous PGE_2 for 0, 4, 6, 24h. Relative expression was determined by dividing expression detected in cells treated with PGE_2 by expression detected in untreated cells. The data are mean ± SEM of n = 3 experiments. * denotes statistical significance relative to the 0h time point ($P < 0.05$).

4.3.7 Differential Gene Expression

Differential gene expression between COX-2 sense and COX-2 antisense cells was investigated using RNA extracted from the two cell lines and the Clontech Atlas human 8K microarrays. Eighty one genes demonstrated differential expression between the two cell lines (see Appendix I) for the complete list of genes demonstrating differential expression).

Interestingly, COX-2 amd growth factors associated with its overexpression such as VEGF were not shown to be differentially expressed. It is plausible that whilst a significant over expression of COX-2 in the COX-2 sense cells relative to the COX-2 antisense cells was demonstrated (see 4.3.1 and 4.3.2), the overall expression of COX-2 and VEGF was below the level of detection of the microarray system. Furthermore, the manufacturers report that a number of false negative and false positive results are observed with the technique.

Of the genes demonstrated to be differentially expressed, one of the greatest differences observed was cathepsin D.

4.3.7.1 Cathepsin D Expression

Cathepsin D mRNA expression demonstrated a 6.7 fold difference between the COX-2 sense and the COX-2 antisense cells as determined by Atlas array analysis; cathepsin D RNA expression was reduced in the COX-2 sense compared with the

COX-2 antisense cells (see figure 4.7 A). Western blot analysis conducted using proteins extracted from the 3 cell lines confirmed lower cathepsin D protein expression in the COX-2 sense and elevated expression in the COX-2 antisense cells compared with the wildtype cells. Cathepsin D protein expression in COX-2 sense and COX-2 antisense was 0.8 ± 0.13 and 1.68 ± 0.39 fold relative to wildtype cells. The difference in cathepsin D expression was 2.16 ± 0.22 fold lower in the COX-2 sense compared with antisense cells $p<0.05$ (see figure 4.7 B and figure 4.7 C).

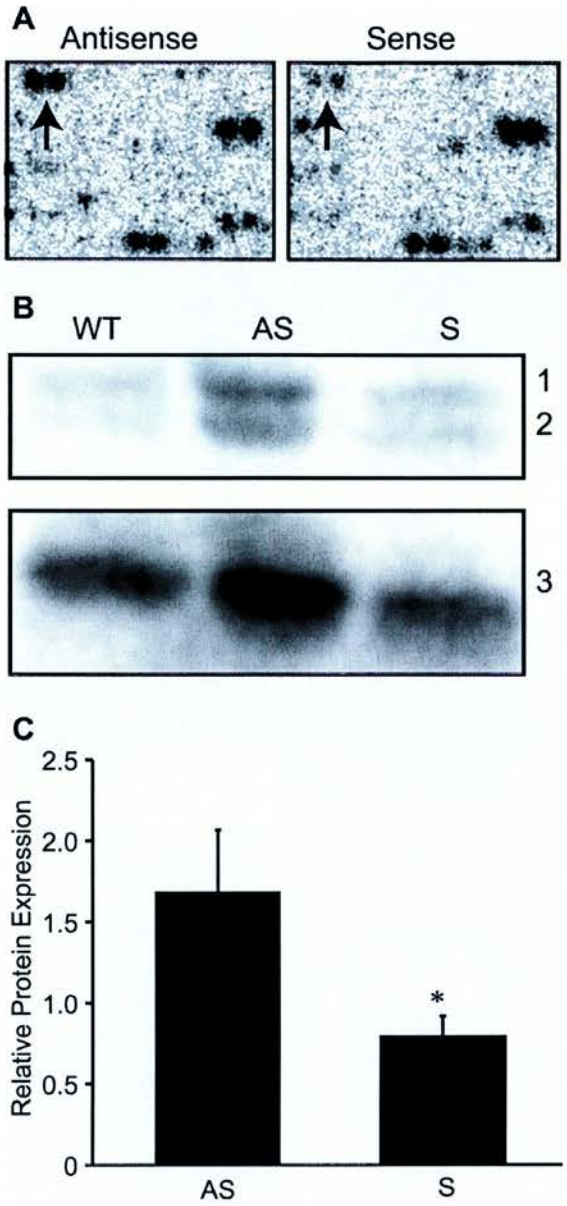

Figure 4.7, **(A)**, Atlas plastic 8K Human cDNA array image following hybridysation with cDNA from untreated COX-2 antisense and COX-2 sense cells. Arrows correspond to cathepsin D position. **(B)**, Western blot analysis of 20 ug protein isolated from untreated Ishikawa wild-type (WT), COX-2 antisense (AS) and COX-2 sense (S) cells. The blot was probed with cathepsin D antibody that detected procathepsin D (1), pseudocathepsin D (2) and cathepsin D (3) protein expression at 52 kDa, 51kDa and 32 kDa respectively. **(C)**, Relative expression of cathepsin D in COX-2 antisense and COX-2 sense cells normalised for β-actin and expressed relative to expression detected in wild-type cells. Bands were semiquantified as outlined and presented as mean ± SEM relative expression of n = 4 experiments. * denotes statistical significance relative to AS ($P < 0.05$).

4.3.8 Cleavage of Plasminogen to Angiostatin

We investigated the potential effect of differential cathepsin D expression in the three cell lines, on cleavage of plasminogen to angiostatin. Plasminogen was cultured in the media collected from COX-2 sense, COX-2 antisense and wild type cells for 0, 4, 8 and 24 hours. Using anti-plasminogen antibody and Western blot analysis, plasmin was detected at 66 kDa and angiostatin bands were detected at 36 and 32 kDa which are within the reported range of 32 – 50 (220, 267). Angiostatin production was elevated at 4, 8 and 24 hours following culture of plasminogen in media collected from COX-2 antisense cells compared with wild type cells. However, angiostatin production was reduced to undetectable levels when plasminogen was incubated in the culture media collected from COX-2 sense cells (see figure 4.8). Interestingly, plasminogen appears to be diminishing after 4 hours in the COX-2 sense cells without being accompanied with angiostatin production. There was no detectable angiostatin production in serum free conditioned media taken from COX-2 sense cells at an earlier time point of 2 hours (data not shown) suggesting it is not due to an earlier production of angiostatin. Furthermore, other studies have demonstrated undetectable angiostatin production by serum free conditioned media cultured with plasminogen is accompanied with an apparent diminished plasminogen level (220).

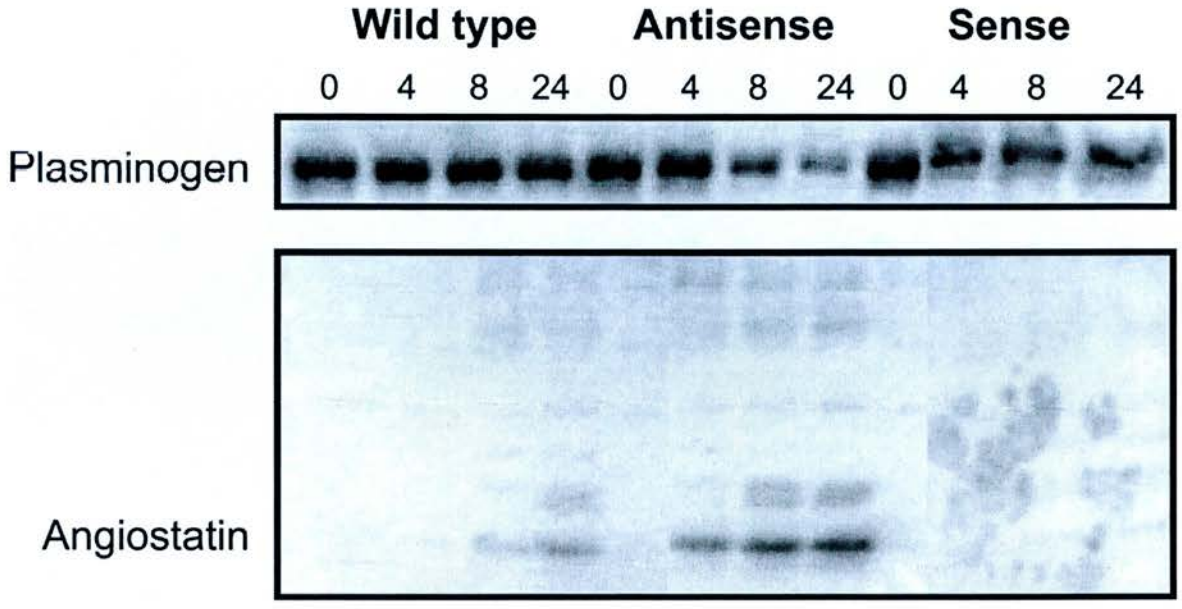

Figure 4.8 Generation of angiostatin following incubation of 25 μg/ml plasminogen for 0, 4, 8, and 24 h in serum – free conditioned media collected from Ishikawa wild – type, COX-2 antisense and COX-2 sense cells. Angiostatin production was detected by Western blot analysis using 20 μl of media. Plasminogen and angiostatin were detected at expected molecular masses of 97 and 36/32 kDa respectively.

4.3.9 Inhibition of Plasminogen Cleavage to Angiostatin by Pepstatin A

In order to confirm that the cleavage of plasminogen to angiostatin is mediated by cathepsin D, the incubation experiments were repeated by incubating plasminogen in COX-2 antisense conditioned media in the presence of the cathepsin D inhibitior pepstatin A. Co- incubation of plasminogen and cathepsin D in the conditioned media from COX-2 antisense cells for 8 hours resulted in inhibition of angiostatin production (see figure 4.9).

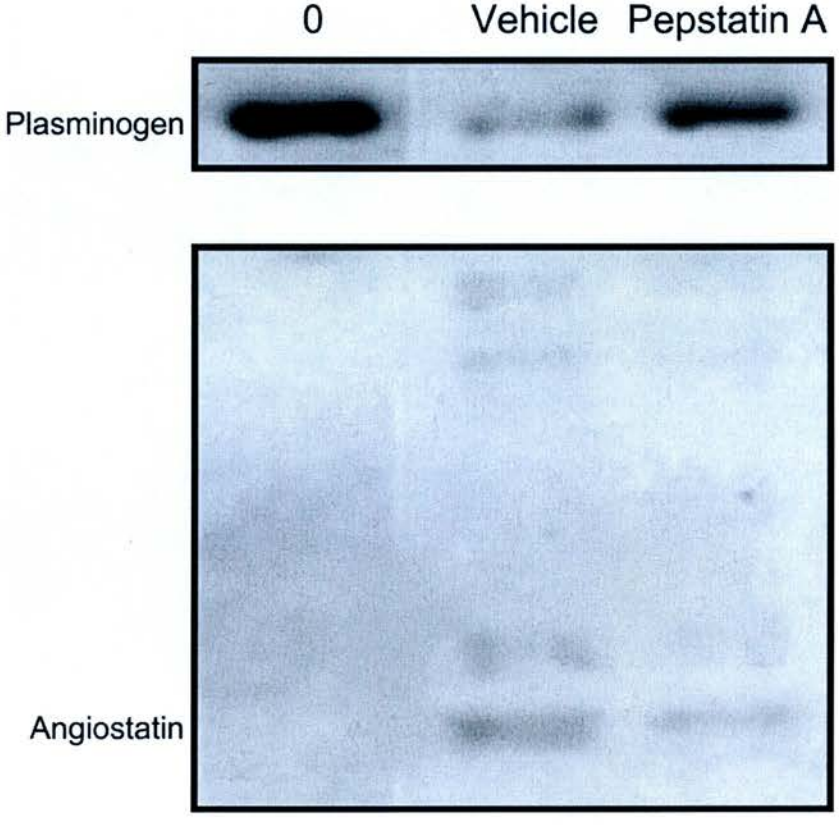

Figure 4.9 Inhibition of angiostatin generation by pepstatin A following incubation of 25 ug/ml plasminogen in media collected from COX-2 antisense cells in the presence or absence of 1 uM pepstatin A for 8h. Angiostatin production was detected by Western blot analysis using 20 ul of media. Plasminogen and angiostatin were detected at expected molecular masses of 97 and 36/32 kDa respectively.

4.4 Discussion

In this study, an endometrial epithelial cell line stably overexpressing COX-2 has been established. COX-2 protein and mRNA expression was higher in the sense clones compared with the antisense and wild type. Overexpression of COX-2 in the Ishikawa endometrial epithelial cells was associated with elevated secretion of PGE_2 and $PGF_{2\alpha}$ that was abolished by the COX-2 selective inhibitor NS398. This is in agreement with other studies that have demonstrated increased prostanoid generation in response to the overexpression of COX-2 (138). Similar expression of COX-1 protein was detected in all three cell lines, suggesting that COX-1 expression is not regulated by COX-2. Treatment of the cells with NS398 reduced PGE_2 and $PGF_{2\alpha}$ secretion in wild-type, COX-2 sense and COX-2 antisense cells and for PGE_2 to almost undetectable levels. This suggests that PGE_2 secretion in the wild-type, COX-2 sense and COX-2 antisense cell lines is predominantly a result of COX-2 activity. COX-2 overexpression was also associated with increased EP2/EP3 receptor expression and signaling with no effect on EP1/EP4 and FP receptor expression. Interestingly, EP4 and FP receptors are known to internalize following treatment with agonist, conversely EP2 and EP3; depending on the isoform, do not display internalization (172, 173, 268-270). To investigate a possible autocrine action of PGE_2 on its EP receptors we measured EP receptor expression in wild type cells following treatment with PGE_2. EP2 mRNA expression was significantly higher following 4 hour treatment of PGE_2 compared with untreated wild type cells. There was no significant difference in EP3 or EP4 mRNA expression. This is in agreement with other studies and suggests PGE_2 acts in an autocrine/paracrine manner to up-

regulate EP2 receptor expression Since PGE_2 did not upregulate EP3 receptor expression in this model, EP3 receptor expression is likely to be regulated by another COX-2 product (271). Furthermore, recent studies have demonstrated cultured media from endometrial epithelial cells upregulate COX-2 and PGE_2 expression in endometrial stromal cells (272). The COX-2 sense cells also displayed significantly higher fold induction of cAMP relative to the COX-2 antisense cells. The EP2 receptor is associated with Gs coupled signaling and results in enhanced cAMP generation (149). The net increase in cAMP in response to exogenous PGE_2 in the COX-2 sense cells suggests an overall balance towards Gs coupled signaling in response to exogenous PGE_2 is favored. Hence, these data confirm that prostanoids such as PGE_2 secreted in response to COX-2 regulate the expression of their receptors and regulate gene expression in an autocrine/paracrine manner.

To identify differential gene expression between the COX-2 sense and the COX-2 antisense cells we employed cDNA array analysis. We demonstrated reduced cathepsin D mRNA and protein expression in the COX-2 sense cells compared with wild type and antisense cells. In the human endometrium, cathepsin D expression has been localised to glandular and stromal cells and glandular expression has been shown to be highest during the secretory phase of the menstrual cycle (273). The elevated expression of cathepsin D is thought to be positively regulated by progesterone. However, cathepsin D expression remains elevated in the mid to late secretory phase despite a reduction in progesterone receptor activity. This has prompted the suggestion that cathepsin D expression is also under the control of other factors (274). Our data demonstrating that cathepsin D expression is inhibited

via a COX-2 mediated action suggests COX-2 may be one of those factors. COX-2 expression in the human endometrium is highest during the late secretory and proliferative phases of the menstrual cycle (23) when cathepsin D levels have been reported to be at their lowest. Interestingly, elevated COX-2 and reduced cathepsin D have independently been associated with a poor prognosis in reproductive tract carcinoma (224, 275, 276). Moreover, COX-2 has been shown to be induced in endometrial adenocarcinomas (61, 62, 257). Hence it is plausible to suggest that increased COX-2 may be an indicator of low cathepsin D expression.

Cathepsin D is known to cleave plasminogen to angiostatin. Angiostatin is a potent endogenous antiangiogenic factor which is proteolytically derived from plasminogen (277). Angiostatin inhibits vasodilation, proliferation and migration of endothelial cells and endothelial tube formation possibly via the induction of apoptosis in endothelial cells (266, 277-280). In human prostate carcinoma cells cathepsin D has been shown to proteolytically cleave plasminogen to angiostatin (215). We initially investigated the generation of angiostatin from plasminogen in conditioned media collected from COX-2 sense, COX-2 antisense cells and wild type. Angiostatin accumulation was reduced to undetectable levels in the media from COX-2 sense cells and elevated in the media from the COX-2 antisense cells compared with the wild type cells supporting a role for cathepsin D in the generation of angiostatin in endometrial epithelial cells. The differential cleavage of plasminogen to cathepsin D in the three cell lines is reflective of the varying degrees of COX-2 expression. Co-incubation of plasminogen in conditioned media from antisense cells with the cathepsin D inhibitor, pepstatin A reduced angiostatin formation. This suggests that

the cleavage of plasminogen to angiostatin is mediated in part by cathepsin D. Other proteolytic enzymes which can cleave plasminogen to angiostatin include matrix metalloproteinases (MMPs) 3,7,9 and 12 (218-220, 281).

Overexpression of COX-2 and prostanoid receptors such as EP2 play a role in angiogenesis by promoting the formation of pro-angiogenic factors (168, 211). Once synthesised the angiogenic factors act in a paracrine manner on endothelial cells to promote enhanced cell migration and tubular formation (138). However, it is now well accepted that the promotion of an angiogenic environment is the result of a balance in the production of angiogenic and anti angiogenic factors (214). The data presented herein demonstrate an alternative pathway by which COX-2 can regulate angiogenesis in endometrial epithelial cells through inhibition of production of antiangiogenic factors. Recently, inhibition of COX-2 has been demonstrated to up regulate the generation and expression of antiangiogenic factors endostatin and thrombospondin-1 (282, 283). Conversely, the data presented herein demonstrate that overexpression of COX-2 inhibits the generation of the antiangiogenic factors such as angiostatin. However, the underlying cellular mechanisms and molecular pathways by which COX-2 down regulates cathepsin D expression resulting in reduced angiostatin generation remains to be elucidated.

In conclusion the data outlined here demonstrate overexpression of COX-2 results in a concomitant induction of PGE_2 secretion and EP2/EP3 receptor expression. COX-2 inhibits cathepsin D expression in endometrial epithelial cells via an unknown mechanism, which contributes to an inhibition of the formation of angiostatin. These

data outline a novel function of COX-2 in promoting a pro angiogenic environment through suppression of production of angiostatin.

Chapter 5

Discussions and Conclusions

5.1 Discussions and Conclusions

As discussed in section 1.7, a role for the cyclooxygenase enzymes and their products the prostaglandins has been described in benign and neoplastic gynaecological conditions. The synthesis of PGE_2 ultimately depends on the actions of the COX enzymes and PGE synthase. Within the human endometrium, previous studies have localised expression of COX enzymes to epithelial and perivascular cells with maximal expression in the menstrual and proliferative phases (23, 102, 241).

Initial studies for this thesis were performed to localise the expression and site of synthesis of PGE synthase and PGE_2 within the human endometrium. Immunohistochemical analysis using paraffin wax-embedded endometrial tissue samples collected from across the menstrual cycle demonstrated mPGES-1 expression and PGE_2 synthesis in endothelial, epithelial and stromal cells of the human endometrium. PGE_2 acts via its G-protein coupled receptors EP1-EP4 (149). Possible sites of action of PGE_2 in the human endometrium were investigated by immunohistochemistry and quantitative RT PCR for two of its receptors namely EP2 and EP4. In various models, PGE_2 acting via its EP2 and EP4 receptors has been associated with elevated expression of angiogenic factors, cellular proliferation and inhibition of apoptosis via a variety of intracellular pathways (168, 284, 285). The endometrium across the menstrual cycle undergoes phases of proliferation and apoptosis with angiogenesis seen throughout the cycle. These studies demonstrated EP2/EP4 receptors localized to glandular epithelial and endothelial cells.

Furthermore, EP2/EP4 receptors displayed temporal upregulation with significantly elevated EP4 receptor expression in the late proliferative phase of the menstrual cycle. EP2/EP4 receptor activation results in the generation of cAMP. Maximal EP4 receptor expression was associated with significantly higher accumulation of cAMP in response to exogenous PGE_2. More recent studies in our laboratory have confirmed the temporal expression and signalling of other COX products namely $PGF_{2\alpha}$ and PGI_2 via their respective FP and IP receptors in the human endometrium (183, 189). These observations further confirm a role for the COX enzymes and prostaglandins in mediating endometrial cell function. However, the role of each of the prostanoid receptors in endometrial physiology remains to be established. Within the human endometrium, the expression of prostaglandins is partly under control of progesterone. Progesterone limits available prostaglandin by positively regulating the expression of the prostaglandin metabolising enzyme; prostaglandin dehydrogenase (PGDH) (286). Progesterone may also inhibit the synthesis of COX-2 (287, 288). There is increasing evidence that the NF$\kappa\beta$ pathway can up regulate COX-2 expression; it is thought the effects of progesterone on COX-2 are mediated via this pathway (107, 108, 289).

In order to investigate further a role for COX-2 in endometrial epithelial cell function, we constructed a stable COX-2 overexpressing cell model in Ishikawa cells which is an endometrial epithelial cell line. The cells were transfected with COX-2 cDNA in either the sense or antisense directions and selected by antibiotic treatment. Overexpression of COX-2 in the COX-2 sense cells was confirmed by Western blot analysis and quantitative RT PCR. Western blot analysis revealed strong COX-2

protein expression in the COX-2 sense cells compared with the Ishikawa wild type and COX-2 antisense cells. Functionality of the transfected COX-2 cDNA was assessed by ELISA to measure PGE_2 and $PGF_{2\alpha}$ synthesis. Both PGE_2 and $PGF_{2\alpha}$ secretion was significantly elevated in the COX-2 sense cells relative to the Ishikawa wild type and COX-2 antisense cells. Furthermore, co-culture of the cells with the specific COX-2 inhibitor, NS398 reduced the increase in PGE_2 and $PGF_{2\alpha}$ secretion. PGE_2 was reduced to almost undetectable levels in the wild-type, COX-2 sense and COX-2 antisense cells suggesting PGE_2 secretion in these cells is predominantly as a result of COX-2 activity.

As mentioned above, PGE_2 and $PGF_{2\alpha}$ act via their G protein coupled receptors, EP1-EP4 and FP respectively. The effect of COX-2 overexpresion on EP1-EP4 and FP receptor expression was assessed by quantitative RT PCR. The relative expression of both EP2 and EP3 receptors was significantly elevated in the COX-2 sense cells compared with the antisense cells suggesting COX-2 products act in an autocrine/ paracrine manner to up-regulate EP receptor expression. This is in agreement with other studies demonstrating overexpression of COX is associated with up regulated EP receptor expression(65, 290). Neither EP4 nor FP receptors were upregulated in the COX-2 sense cells. In other in vitro models, overexpression of COX enzymes has been associated with elevated EP4 receptors (65). Previous studies have demonstrated that EP4 and FP receptors both undergo internalisation upon agonist activation (172, 269). In this overexpressing COX-2 model, internalisation of EP4 and FP receptors thus reducing the number of ligand binding

sites may be acting to prevent an up regulation of these receptors by an autocrine action, thereby maintaining the receptor expression at basal level.

The enhanced expression of EP2 and EP3 receptors was concomitant with a net increase in cAMP in response to exogenous PGE_2 in the COX-2 sense cells relative to the Ishikawa wild type and COX-2 antisense cells. EP2 receptors signal via Gs. However, there are multiple EP3 splice variants that are coupled with Gs or Gi. These data suggest that in our model system, an overall balance toward Gs coupled signalling in response to PGE_2.

A possible autocrine effect of PGE_2 on its EP receptor expression was confirmed by the upregulation of EP2 receptors in response to PGE_2 treatment in Ishikawa wild type cells, pre-treated with the non selective COX inhibitor indomethacin. This autocrine/paracrine effect of PGE_2 on EP receptor expression is supported by other studies demonstrating PGE_2 upregulates EP2 receptor expression with no effect on EP4 (271). Interestingly, PGE_2 has also been shown to enhance COX-2 expression probably via a cAMP dependent pathway (291). It is suggested that PGE_2 augments its actions in a feed forward mechanism not only by regulating the expression of its receptors but also by modulating the expression of the rate limiting enzyme (COX-2) in its synthesis.

In order to determine a role for COX-2 in endometrial epithelial cells, cDNA array technology was employed to determine which genes were differentially regulated between the COX-2 sense and the COX-2 antisense cells. Cathepsin D was identified

as one of the differentially expressed genes. Cathepsin D mRNA and protein expression were reduced in the COX-2 sense cells compared with the COX-2 antisense cells. In the endometrium, glandular expression of Cathpesin D is highest during the secretory phase of the menstrual cycle and thought to be positively controlled in part by progesterone (273, 274). These data suggests COX-2 may be another regulatory factor of cathepsin D within the human endometrium.

Angiostatin is a potent anti-angiogenic factor and one of a number of endogenous antiangiogenic factors; including endostatin and thrombospondin-1. Angiostatin is proteolitically derived from plasminogen and contains the first 3 or 4 kringle domains of plasminogen (277). Plasminogen is initially cleaved to plasmin which can act in a pro-angiogenic manner to promote endothelial cell migration (292). Further cleavage results in the formation of angiostatin which displays antiangiogenic behaviour. Angiostatin inhibits vasodilation, proliferation and migration of endothelial cells (277-280, 293). The mechanisms by which angiostatin inhibits angiogenesis are not fully understood. By binding to the integrin $\alpha v\beta 3$ receptor, angiostatin inhibits plasmin mediated cell migration (294). Angiostatin has also been shown to inhibit the phosphorylation of ERK1/2 by hepatocyte growth factor (295). Other binding sites for angiostatin that have been identified include the F_1-F_0 ATP synthase expressed on endothelilal cell surface and angiomotin (296, 297). There is also evidence that angiostatin can directly affect tumour cell function by the down regulation of VEGF expression (298).

Conditioned medium of human prostate carcinoma cells has been demonstrated to cleave plasminogen to angiostatin by the actions of mature and pro-cathepsin D (215). In our COX-2 overexpressing model, angiostatin accumulation in media was elevated in the COX-2 antisense cell and abolished in the COX-2 sense cells relative to Ishikawa wild type cells. Inhibition of cathepsin D with pepstatin A reduced angiostatin generation in the media from COX-2 antisense cells. These data support a role in part for cathepsin D in generating angiostatin.

Angiostatin was originally identified in the urine and serum of tumour baring mice. Most studies to date have investigated angiostatin expression in pathological states. Recently, the expression of angiostatin was identified in healthy gingiva by immunohistochemistry (299). This gives rise to the suggestion that angiostatin is endogenously produced in non tumour environments.

To date, no studies have investigated the expression of angiostatin within the normal endometrium, however, thrombospondin-1 expression has been demonstrated and shown to inhibit the migration of endothelial cells. Furthermore, the expression of thrombospondin-1 was restricted to the secretory phase of the menstrual cycle and its expression shown to be positively linked to progesterone (300). As discussed previously, COX expression in the human endometrium is lower during the secretory phase than the menstrual and proliferative phases of the menstrual cycle. Although currently there is no evidence to support the idea, it is plausible that COX-2 may play a role in the regulation of thrombospondin-1 expression in the endometrium. Endostatin is the cleaved product from collagen XVIII (301). Inhibition of COX-2

has been associated with up regulated expression of endostatin (282). Interestingly, endostatin itself has been shown to inhibit the mRNA expression of growth factors such as VEGF, NF$\kappa\beta$ and COX-2 and up regulate thrombospondin-1 (302). In patients with endometrial cancer, the ratio of VEGF: endostatin was shown to be elevated in late stages of the disease compared with disease free subjects (303). Further circumstantial evidence of a role for COX-2 in the regulation of endostatin comes from the observation that hypoxia which has been demonstrated to induce COX-2 expression is associated with elevated VEGF expression and an inhibition of endostatin generation (304, 305).

It is now well accepted that the promotion of an angiogenic environment is the result of the balance in production of angiogenic and antiangiogenic factors (214). The underlying molecular mechanisms in the control of endogenous formation of antiangiogenic factors and their mechanisms of action have yet to be elucidated. It is hypothesised that in gynaecological conditions associated with elevated COX-2 enzyme expression, vascular function may be promoted through overexpression of angiogenic factors and reduced production of antiangiogenic factors such as angiostatin. Furthermore, localised modulation of antiangiogenic factors including angiostatin within the human endometrium may provide alternative therapies for benign gynaecological conditions associated with aberrant vascular function. In a murine model of endometriosis, transient overexpression of angiostatin, in the peritoneum delivered by a replication-deficient adenovirus vector eradicated established endometriosis (306).

5.2 Conclusions and Future Studies

In conclusion, this thesis has demonstrated the temporal expression and signalling of the prostaglandin E_2 pathway in the normal human endometrium across the menstrual cycle. PGE_2, mPGES-1 and EP2/EP4 receptors have been localised to multiple cell types in the human endometrium. Overexpression of COX-2 in an endometrial epithelial cell line results in an induction of PGE_2 and $PGF_{2\alpha}$ expression concomitant with elevated EP2 and EP3 receptor expression. Furthermore, a possible autocrine action of PGE_2 on its EP receptor expression has been identified. COX-2 inhibits cathepsin D via an unknown mechanism which contributes to an inhibition of the formation of the antiangiogenic factor angiostatin. These data outline a novel function of COX-2 in promoting a proangiogenic environment through the suppression of production of antiangiogenic factors such as angiostatin. Thus in a pathological state, enhanced expression of COX-2 may not only promote angiogenesis via the upregulation of pro angiogenic factors as seen by other studies (138, 168, 207, 209), but could augment this action by inhibiting the production of endogenous antiangiogenic factors.

This thesis identified a novel function of COX-2 in maintaining a pro angiogenic environment by inhibiting cathepsin D expression and subsequent production of the antiangiogenic factor angiostatin. The mechanism and prostanoid receptors involved in the regulation of cathepsin D by COX-2 have yet to be elucidated.

As described in section 1.19, this thesis sought to explore the hypothesis that COX-2 and its products the prostaglandins play a role in endometrial vascular function. PGE2 was localised to various cells of the human endometrium including endothelial cells. Overexpression of COX-2 in endometrial epithelial cells resulted in reduced angiostatin; an antiangiogenic factor, generation. An enhanced production of growth factors such as VEGF has previously been reported in COX-2 overexpression models. Interestingly, the expression of VEGF mRNA as investigated by microarray technology was not upregulated by the overexpression COX-2 in Ishikawa cells, an endometrial epithelial cell line. As discussed in section 4.3.7 it is plausible this is due to the limitations of the technique. Further studies using alternative techniques such as ELISA to examine VEGF protein expression are needed to investigate further the role of COX-2 overexpression on VEGF and other pro angiogenic growth factor expression in endometrial epithelial cells. As detailed in section 5.2.2, further studies are also required to investigate the expression of angiostatin by the endometrium. This thesis, however, identified a novel putative role for COX-2 and the prostaglandins in promoting endometrial vascular function by reducing antiangiogenic factor generation.

5.2.2 Future Studies

The profile of prostanoid receptors expressed in benign endometrial pathologies compared with the normal endometrium has yet to be established. If aberrant receptor expression in endometrial pathologies is confirmed, this will give an insight into the receptors which contribute to endometrial pathology. Furthermore, selective

inhibition of receptor activity may abolish the vascular dysfunction thought to be associated with benign gynaecological conditions. Previous studies in our laboratory have demonstrated the upregulation of EP and FP receptor expression and signalling in endometrial and cervical carcinomas (60, 62, 187). These receptors have been shown to induce the expression of the angiogenic factors VEGF in the female reproductive tract (168). This has led to the suggestion that inhibition of specific prostaglandin receptors could be a mode for therapy in gynaecological conditions. EP receptor signalling has been demonstrated to play an important function in colorectal carcinogenesis with the specific EP receptor activity varying according to the stage of tumorigenesis. Furthermore, it is thought that inhibition of the EP receptors may provide an important therapeutic role in colorectal cancer (307).

As described previously, there are a host of endogenous antiangiogenic factors including angiostatin, endostatin and thrombospondin-1. The expression of these factors in non pathological states has not been fully determined. The endometrium is unusual in the fact it regularly undergoes angiogenesis. Characterisation of the expression of antiangiogenic factors and the processes involved in the regulation of their expression in the normal endometrium and the molecular pathways by which antiangiogenic factors mediate their effects will provide deeper understanding of the control of physiological angiogenesis.

Finally, characterisation of prostanoid receptor expression in benign gynaecological conditions relative to the normal endometrium and their role in the control of pro angiogenic and antiangiogenic factors would ultimately lead to the identification of

novel targets for therapies. This would hopefully result in improved treatment for benign and neoplastic pathologies involving aberrant vascular function.

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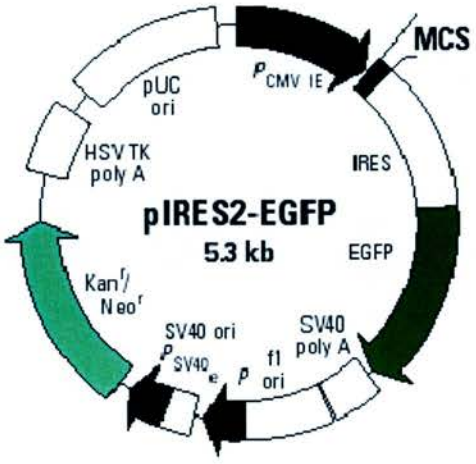
Appendix I Genes differentially regulated by the overexpression of COX-2, the ratios is the relative RNA expression in COX-2 sense cells relative to COX-2 antisense cells

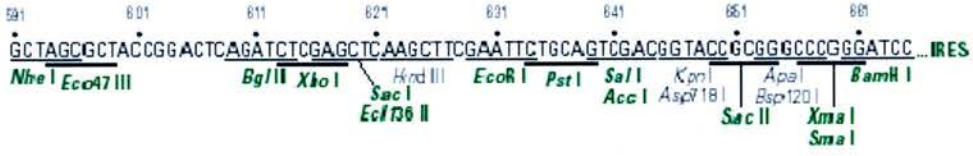
RATIO UP	RATIO DOWN	Gene	Functional Group
2.2		lymphocyte antigen 6 complex, locus H	Cell surface antigens
2.1		transforming growth factor beta 1 induced transcript 1	transcription
2.4		TG-interacting factor (TALE family homeobox)	transcription
2.9		slug (chicken homolog), zinc finger protein	transcription
3.3		early growth response 1	transcription
3.0		nuclear receptor coactivator 3	transcription
	3.0	DNA-damage-inducible transcript 3	transcription
	2.1	interleukin enhancer binding factor 3, 90kD	cell cycle
	2.7	cyclin I	cell cycle
	2.1	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	cell cycle
3.1		integrin, beta 4	cell adhesion receptors / proteins
	4.7	laminin receptor 1 (67kD, ribosomal protein SA)	cell adhesion receptors / proteins
2.0		Fc fragment of IgG binding protein	extracellular transport / carrier proteins
	3.2	lectin, galactoside-binding, soluble, 1 (galectin 1)	extracellular transport/carrier proteins,
	2.4	phospholipid transfer protein	extracellular transport/carrier proteins
	2.6	proline-rich protein BstNI subfamily 3	extracellular transport/carrier proteins
2.7		breast cancer anti-estrogen resistance 1	Oncogene and tumour supressor
	3.5	exostoses (multiple)-like 1	Oncogene and tumour supressor
	3.2	heat shock 90kD protein 1, beta	Stress response
2.1		heat shock 70kD protein 8	Stress response
	2.1	aquaporin 8	Membrane channels and transporters
	2.8	potassium inwardly-rectifying channel, subfamily J, member 3	Membrane channels and transporters

6.3		solute carrier family 34 (sodium phosphate), member 2	Membrane channels and transporters
	2.4	mitochondrial solute carrier	Membrane channels and transporters
3.2		ladinin 1	Extracellular matrix proteins
6.3		collagen, type VI, alpha 1	Extracellular matrix proteins
2.1		tectorin alpha	Extracellular matrix proteins
2.7		collagen, type I, alpha 2	Extracellular matrix proteins
	3.7	cargo selection protein (mannose 6 phosphate receptor binding protein)	Trafficking / targetting
2.2		cellular retinoic acid-binding protein 1	Trafficking / targetting
	2.1	dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit	Trafficking / targetting
2.1		cytochrome c oxidase subunit VIIb	metabolism
	2.1	hemoglobin, theta 1	metabolism
	2.1	cytochrome c oxidase subunit VIb	metabolism
5.4		triosephosphate isomerase 1	metabolism
2.2		phosphorylase, glycogen; brain	metabolism
2.0		glyceraldehyde-3-phosphate dehydrogenase	metabolism
	2.2	glutamate-ammonia ligase (glutamine synthase)	metabolism
	2.0	protein kinase, AMP-activated, beta 1 non-catalytic subunit	metabolism
7.3		phosphatidic acid phosphatase type 2B	metabolism
2.2		hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	metabolism
	2.2	aldehyde dehydrogenase 3	metabolism
	2.1	chaperonin containing TCP1, subunit 5 (epsilon)	post translational modification / protein folding
	3.2	integral membrane protein 1	post translational modification / protein folding
2.1		ribosomal protein S4, X-linked	Translation
	2.0	ribosomal protein L3	Translation
	2.2	Tu translation elongation factor, mitochondrial	Translation
2.1		death receptor 6	Apoptosis associated proteins
2.0		BCL2-like 1	Apoptosis associated proteins
	2.3	HLA-B associated transcript-1	RNA processing tunover and transport
	3.3	H2A histone family, member X	DNA binding and chromatin proteins
	2.0	high-mobility group (nonhistone chromosomal) protein 17	DNA binding and chromatin proteins
	2.1	neurotrophic tyrosine kinase, receptor, type 1	Cell receptors
	2.0	Duffy blood group	Cell receptors

2.1		melatonin receptor 1B	Cell receptors
2.0		oncostatin M	Cell signalling, extracellular communication proteins
3.5		cysteine-rich, angiogenic inducer, 61	Cell signalling, extracellular communication proteins
	3.7	casein kinase 2, beta polypeptide	Intracellular transducers / effectors / modulators
	2.0	flotillin 1	Intracellular transducers / effectors / modulators
	5.8	amyloid beta (A4) precursor-like protein 2	Intracellular transducers / effectors / modulators
2.5		Arg/Abl-interacting protein ArgBP2	Intracellular transducers / effectors / modulators
3.4		tuberous sclerosis 2	Intracellular transducers / effectors / modulators
6.6		protease, serine, 2 (trypsin 2)	Protein turnover
	6.7	cathepsin D (lysosomal aspartyl protease)	Protein turnover
2.4		matrix metalloproteinase 15 (membrane-inserted)	Protein turnover
	2.1	proteasome (prosome, macropain) subunit, beta type, 3	Protein turnover
2.0		G protein-coupled receptor 25	Cell receptors
	2.1	G protein-coupled receptor 26	Cell receptors
	2.1	keratin 16 (focal non-epidermolytic palmoplantar keratoderma)	Cytoskeleton / motility proteins
2.6		keratin 18	Cytoskeleton / motility proteins
2.1		cytoplasmic linker 2	Cytoskeleton / motility proteins
	2.0	moesin	Cytoskeleton / motility proteins
	2.8	complement component 1, q subcomponent binding protein	Functionality unclassified
2.0		melanoma antigen, family A, 2	Functionality unclassified
2.4		neuron-specific protein	Functionality unclassified
2.1		tubby like protein 3	Functionality unclassified
2.4		brain specific protein	Functionality unclassified
	2.1	ART-4 protein	Functionality unclassified
2.3		chromosome 1 open reading frame 2	Functionality unclassified
	2.6	non-functional folate binding protein	Functionality unclassified
	6.8	tumor suppressing subtransferable candidate 3	Functionality unclassified
	2.3	NICE-5 protein	Functionality unclassified
2.1		BLu protein	Functionality unclassified

Appendix II





The pIRES2 vector, the gene of interest was cloned into the multiple cloning site (MCS). Adapted from the Clontech web site (www.clontech.com).

Appendix III

The following publications and conference proceedings have arisen from this thesis:

PUBLICATIONS

SA Milne, GB Perchick, SC Boddy and HN Jabbour. (2001). Expression, Localisation, and signaling of PGE_2 and EP2/EP4 Receptors in human nonpregnant endometrium across the menstrual cycle. Journal of Clinical Endocrinology and Metabolism. 86(9). 4453-4459.

GB Perchick and HN Jabbour. (2003) Cyclooxygenase-2 overexpression inhibits cathepsin D-mediated cleavage of plasminogen to the potent antiangiogenic factor angiostatin. Endocrinology. 144(12). 5322-5328

CONFERENCE PROCEEDINGS

J Brooks, GB Perchick, SC Boddy, RA Anderson and HN Jabbour. (2000) Temporal expression of prostaglandin EP2 and EP4 receptors in human endometrium during the menstrual cycle Society for the Study of Fertility, Edinburgh, UK. Journal of Reproduction and Fertility, abstract series 25:177.

GB Perchick and HN Jabbour (2001). PGE_2 and $PGF_{2\alpha}$ secretion in endometrial epithelial cells stably overexpressing COX-2 Society for the Study of Fertility, Cambridge, UK. Reproduction, abstract series 27:101.

GB Perchick and HN Jabbour (2002).Enhanced PGE_2 Secretion and EP2 Receptor Expression and Signalling in Endometrial Epithelial Cells Stably Overexpressing COX-2. 84^{th} Annual Meeting of the Endocrine Society, San Francisco, OR-36-6

GB Perchick and HN Jabbour (2003). COX-2 reduces the cleavage of plasminogen to the potent antiangiogenic factor angiostatin Society for the Study of Fertility, Aberdeen, UK. Reproduction, abstract series 30:O

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Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 86(9):4453–4459

Expression, Localization, and Signaling of PGE_2 and EP2/EP4 Receptors in Human Nonpregnant Endometrium across the Menstrual Cycle

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This study was designed to elucidate the sites of synthesis and action of PGE_2 in the nonpregnant human uterus across the menstrual cycle. The sites of expression of PGE synthase and synthesis of PGE_2 were investigated by immunohistochemistry using full thickness uterine biopsies. Expression of PGE synthase and synthesis of PGE_2 were localized to glandular epithelial and endothelial cells in both basalis and functionalis regions of the human endometrium. By contrast, stromal staining was predominantly localized in the functionalis layer. Some cyclical variation in expression of PGE synthase and PGE_2 synthesis was observed, with reduced expression/synthesis detected in the stromal compartment of the functionalis during the late secretory phase of the menstrual cycle. Subsequently, we assessed the site of action of PGE_2 by investigating the expression of two PGE_2 receptor isoforms, namely EP2 and EP4. Cyclical variation in endometrial EP2 and EP4 receptor mRNA expression was quantified by TaqMan quantitative RT-PCR using RNA isolated from endometrial tissue collected across the menstrual cycle. No differences in EP2 receptor mRNA expression were detected; however, EP4 receptor mRNA expression was significantly higher in late proliferative stage ($P < 0.05$) than in early, mid, and late secretory stage endometrium. Expression patterns of EP2 and EP4 receptors were localized by nonradioactive *in situ* hybridization using fluorescein isothiocyanate end-labeled oligonucleotide probes. Expression of both receptors was observed in endometrial glandular epithelial and vascular cells, with no notable spatial or temporal variation. Finally, signaling of EP2/EP4 receptors was assessed by investigating cAMP generation *in vitro* after stimulation with PGE_2. Endometrial cAMP generation in response to PGE_2 was significantly greater in proliferative tissue compared with early and midsecretory stage tissue (3.77 ± 0.85 *vs.* 1.96 ± 0.28 and 1.38 ± 0.23, respectively; $P < 0.05$). In conclusion, this study demonstrates glandular and vascular coexpression of PGE synthase, PGE_2, EP2, and EP4 receptors and suggests an autocrine/paracrine role for PGE_2 in epithelial/endothelial cell function in the human endometrium. (*J Clin Endocrinol Metab* 86: 4453–4459, 2001)

PGs ARE part of the eicosanoid family and consist of five endogenous members, termed PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin, and thromboxane A_2 (1). Initially in the PG synthesis pathway, the enzyme cyclooxygenase (COX) generates PGH_2 from arachidonic acid. To date, there are two identified isoforms of the COX enzyme, COX-1 and COX-2. COX-1 is constitutively expressed in many tissues and cell types and generates PG for normal physiological function. By contrast, the expression of COX-2 is rapidly induced after the stimulation of quiescent cells by growth factors and phorbol esters (2). Once synthesized, PGH_2 serves as a substrate for specific PG synthase enzymes (3–7). The PG synthase enzymes are named according to the PG they produce, such that PGE_2 is generated by PGE synthase and $PGF_{2\alpha}$ by PGF synthase. Once synthesized, PG mediate their actions via seven-transmembrane G protein-coupled receptors. These G protein-coupled receptors have been cloned in humans and are denoted DP, EP, FP, IP, and TP according to their endogenous PG ligand (1, 8). Only the EP receptor has been shown to possess diverse receptor subtypes, of which there are now four members, termed EP1, EP2, EP3, and EP4. EP1 receptors activate phospholipase C and mobilization of the inositol trisphosphate pathway, EP2 and EP4 receptors activate adenylate cyclase and the cAMP/protein kinase A pathway, whereas EP3 receptor activation can both inhibit adenylate cyclase and activate phospholipase C (1).

Recent studies have demonstrated a role for COX enzymes and PG in the regulation of epithelial cell growth and angiogenesis. COX-2 expression and PGE_2 synthesis are associated with increased cellular proliferation and resistance to apoptosis (9, 10). Moreover, expression of COX-2 and synthesis of PGE_2 in epithelial cells enhance the expression of angiogenic factors that act in a paracrine manner to induce endothelial cell migration and microvascular tube formation (11). COX-2 expression and PGE_2 synthesis have also been associated directly with endothelial cell function (12). Treatment of endothelial cells with selective COX-2 inhibitors has been shown to reduce microvascular tube formation, and this effect is partially reversed by cotreatment with PGE_2 (11, 13).

In the human endometrium, COX-2 enzyme expression and PGE_2 synthesis have been associated with vascular function. COX enzyme expression and PGE_2 synthesis are maximal during the menstrual and proliferative phases and are localized to epithelial and perivascular cells (14–18). Further evidence for a role for PGE_2 in vascular function of the endometrium can be deduced from observations in women suffering from excessive blood loss at menstruation (menorrhagia). In this group of women, a positive relationship between the volume of blood loss and PGE_2 release *in utero* has been reported (19).

Abbreviations: COX, Cyclooxygenase; dNTP, deoxy-NTP; FITC, fluorescein isothiocyanate; PG, prostaglandin.

The aims of this study were to characterize the site of synthesis and action of PGE_2 in human endometrium across the menstrual cycle. This was assessed by investigating the cyclical changes in expression, localization, and functional signaling of PGE synthase, PGE_2, and two PGE receptors, namely EP2 and EP4. The data from this study demonstrate epithelial and endothelial expression/synthesis of PGE synthase, PGE_2, and EP2 and EP4 receptors in the human endometrium. Furthermore, cAMP production in response to PGE_2 is higher in proliferative phase than secretory phase endometrium. These data strongly suggest a role for PGE_2 in uterine epithelial cell function and angiogenesis, possibly through autocrine/paracrine signaling between endometrial epithelial and endothelial cells.

Materials and Methods

Patients and tissue collection

Endometrial biopsies (n = 33) at different stages of the menstrual cycle were collected with an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France) from women with regular menstrual cycles (25–35 d). In addition, full thickness endometrial biopsies (n = 18) at all stages of the menstrual cycle (n = 3 from early, mid, and late proliferative and n = 3 from early, mid, and late secretory) were collected from women undergoing hysterectomy for benign gynecological indications. Shortly after pipelle suction or hysterectomy, tissue was either snap-frozen in dry ice and stored at −70 C (for RNA extraction), fixed in neutral buffered formalin, and wax embedded (for immunohistochemical analyses) or placed in RPMI 1640 (containing 2 mmol/liter L-glutamine, 100 U penicillin, and 100 μg/ml streptomycin) and transported to the laboratory for *in vitro* culture. All subjects reported regular menstrual cycles (cycle length, 25–35 d), and no woman had received a hormonal preparation in the 3 months preceding biopsy collection. Biopsies were dated according to stated last menstrual period and were confirmed by histological assessment according to criteria of Noyes and co-workers (20). Furthermore, circulating E2 and progesterone concentrations at the time of biopsy were consistent for both stated last menstrual period and histological assignment of menstrual cycle stage. Ethical approval was obtained from Lothian research ethics committee, and written informed consent was obtained from all subjects before tissue collection.

Immunohistochemistry

Endometrial sections (5 μm) from across the menstrual cycle (n = 18) were dewaxed in xylene and rehydrated using decreasing grades of ethanol. An antigen retrieval step of 5 min of pressure cooking in 0.01 M sodium citrate buffer, pH 6.0, was carried out for PGE synthase. After rinsing in PBS, endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. Nonimmune swine serum (10% serum in PBS) was applied for 20 min before overnight incubation at 4 C with primary antibody. An avidin-biotin peroxidase detection system was then applied (DAKO Corp., High Wycombe, UK) with 3,3′-diaminobenzidine as the chromagen. Sections were counterstained with Harris's hematoxylin before mounting. Rabbit antihuman PGE synthase antibody (Cayman Chemicals, Ann Arbor, MI) was used at a 1:250 dilution, and rabbit anti-PGE_2 antibody (supplied by Prof. R. W. Kelly, Medical Research Council Human Reproductive Sciences Unit, Edinburgh, UK) was used at a dilution of 1:100. Preabsorption with PGE synthase-blocking peptide (100 μg/ml: Cayman Chemicals) was the PGE synthase negative control, and preabsorption with excess PGE_2 was the PGE_2 negative control. Both preabsorbed antibody preparations produced negligible immunoreactivity. All treatments were carried out at room temperature unless otherwise specified. The immunohistochemistry was repeated for each antibody at least three times.

In situ hybridization

Custom synthesis oligonucleotide double fluorescein isothiocyanate (FITC)-labeled cDNA probes for EP2 and EP4 receptor were obtained from Biognostik (Gottingen, Germany). Sections (5 μm) were cut onto gelatin-coated SuperFrost slides (BDH Laboratory Supplies, Poole, UK) from full thickness human uterine biopsies collected across the menstrual cycle (n = 18). Tissue was dewaxed in xylene and rehydrated using increasing concentrations of ethanol before proteinase K treatment (100 μg/ml in 100 mM Tris-HCl pH 7.6, containing 50 mM EDTA) for 15 min at 37 C to enhance cDNA probe access. After washing in diethylpyrocarbonate-H_2O, hybridization mixture (25 μl; supplied with probe) was added to each section, and slides were incubated for 4 h at 30 C before adding cDNA probe (6 U/ml hybridization mix) and incubating overnight at 30 C. Posthybridization washes of 1 × SSC (standard saline citrate) for 5 min (twice) and 0.1 × SSC at 39 C for 15 min (twice) were completed before detecting the FITC-labeled probe using standard immunohistochemical reagents (TSA Biotin System, NEN Life Science Products, Hounslow, UK). Endogenous peroxidase activity was first blocked with 3% H_2O_2 in methanol for 30 min before incubating sections with blocking buffer for 30 min. Conjugated anti-FITC-horseradish peroxidase (Roche Molecular Biochemicals, Mannheim, Germany) was added in blocking buffer, and the sections were incubated for 60 min. After washing, biotinyl tyramide amplification reagent was applied to each slide and incubated for 15 min. Streptavidin-horseradish peroxidase was applied after washing and incubated for 30 min, and probe localization was visualized with 3,3′-diaminobenzidine. Control oligonucleotide double FITC-labeled cDNA probes containing the same proportion of cysteine (C) and guanine (G) bases as the EP2 and EP4 receptor probes were included to assess background hybridization. All treatments were carried out at room temperature unless otherwise specified.

Taqman quantitative RT-PCR

Endometrial RNA samples were extracted from endometrial biopsies (n = 33) using Tri-Reagent (Sigma, Poole, UK) following the manufacturer's guidelines. Once extracted and quantified, RNA samples were reverse transcribed using $MgCl_2$ (5.5 mM), deoxy (d)-NTPs (0.5 mM each), random hexamers (2.5 μM), ribonuclease inhibitor (0.4 U/μl), and multiscribe reverse transcriptase (1.25 U/μl; all from Applied Biosystems, Warrington, UK). The mix was aliquoted into individual tubes (16 μl/tube), and template RNA was added (4 μl/tube of 100 ng/μl RNA). After mixing by brief centrifugation, samples were incubated for 90 min at 25 C, 45 min at 48 C, and 5 min at 95 C. Thereafter, cDNA samples were stored at −20 C. A tube with no reverse transcriptase was included to control for any DNA contamination.

To measure cDNA expression, a reaction mix was prepared containing TaqMan buffer (5.5 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, and 400 μM dUTP), ribosomal 18S forward and reverse primers and probe (all at 50 nM), forward and reverse primers for EP receptor (300 nM), EP receptor probe (100 nM), AmpErase UNG (0.01 U/μl), and AmpliTaq Gold DNA Polymerase (0.025 U/μl; all from Applied Biosystems). After mixing, 48 μl were aliquoted into separate tubes, and 2 μl/replicate (40 ng) cDNA were added and mixed before placing duplicate 24-μl samples into a PCR plate. A no template control (containing water) was included in triplicate. Wells were sealed with optical caps, and the PCR reaction was carried out using an ABI Prism 7700 (Applied Biosystems). EP receptor primers and probe for quantitative PCR were designed using the PRIMER express program (Applied Biosystems). The sequences of the EP2 receptor primers and probe were: forward, 5′-GAC CGC TTA CCT GCA GCT GTA C-3′; reverse, 5′-TGA AGT TGC AGG CGA GCA-3′; and probe (6-carboxy fluoroscein labeled), 5′-CCA CCC TGC TGC TGC TTC TCA TTG TCT-3′. The sequences of the EP4 receptor primers and probe were: forward, 5′-ACG CCG CCT ACT CCT ACA TG-3′; reverse, 5′-AGA GGA CGG TGG CGA GAA T-3′; and probe (6-carboxy fluoroscein labeled), 5′-ACG CGG GCT TCA GCT CCT TCC T-3′. The ribosomal 18S primers and probe sequences were: forward, 5′-CGG CTA CCA CAT CCA AGG AA-3′; reverse, 5′-GCT GGA ATT ACC GCG GCT-3′; and probe (VIC labeled), 5′-TGC TGG CAC CAG ACT TGC CCT C-3′. Data were analyzed and processed using Sequence Detector version 1.6.3 (Applied Biosystems) as instructed by the manufacturer. Briefly, the software calculates the reaction cycle number at which fluorescence reaches a determined level for both 18S control and EP2/EP4 receptor. This is the relative abundance for EP2/EP4 receptor, and by comparing this to an internal positive control, relative expression can be determined. Results are expressed as relative expression to an internal positive standard included in all reactions.

Whole tissue cAMP assay

Endometrial biopsies from all stages of the menstrual cycle (n = 18; n = 6 from proliferative, early, and midsecretory phases) were minced finely with scissors and incubated in 2 ml RPMI (Sigma) medium containing 10% FCS, 2 mmol/liter L-glutamine, 100 IU penicillin, 100 μg streptomycin, and 3 μg/ml indomethacin for 1.5 h at 37 C in a humidified 5% CO_2 incubator. Tissue specimens were incubated in the same medium containing isobutylmethylxanthine (Sigma) to a final concentration of 1 mM for 30 min at 37 C before adding 300 nM PGE_2. Control tissue was treated similarly, but received no PGE_2. Tissue was harvested by centrifugation at 2000 × *g*, the supernatant was discarded, and the tissue was homogenized in 0.1 M HCl. The cAMP concentration was quantified by ELISA using cAMP kits (Biomol, Affiniti, Exeter, UK) and was normalized to protein concentration of the homogenate. Protein concentrations were determined using protein assay kits (Bio-Rad Laboratories, Inc., Hemel Hempsted, UK). Data are presented as the fold induction of cAMP after treatment with PGE_2, where fold induction was calculated relative to the control samples.

Statistics

Where appropriate, data were subjected to statistical analysis with ANOVA and Fisher's protected least significant difference tests (StatView 4.0, Abacus Concepts, Inc., Berkeley, CA), and statistical significance was accepted at $P < 0.05$.

Results

Spatial localization of PGE synthase in full thickness endometrial biopsies demonstrated marked differences in PGE synthase expression between distinct uterine regions (Fig. 1, a–f). In the functionalis layer, PGE synthase immunoreactivity was expressed in glandular epithelial, stromal, and endothelial cells (Fig. 1, b–e). Temporally, within the functionalis layer PGE synthase immunoreactivity only varied in the late secretory phase, where reduced staining of stromal cells was observed (Fig. 1e). Compared with the functionalis region, little stromal staining for PGE synthase was detected in the basalis compartment throughout the menstrual cycle; however, comparable immunoreactivity for PGE synthase was observed in epithelial and endothelial cells of this region (Fig. 1a). In the myometrial region, only endothelial cells were immunopositive for PGE synthase (Fig. 1f, *inset*). When viewed as a cross-section, PGE synthase was expressed in all epithelial and endothelial cells throughout the uterus, but stromal cell immunoreactivity diminished from strong staining in the functionalis layer to minimal immunoreactivity in the myometrial fibroblasts (Fig. 1f).

Within cells, particularly functionalis stromal cells, PGE synthase immunoreactivity was predominantly cytoplasmic, with strong perinuclear localization (Fig. 1, b and c). Perinuclear PGE synthase expression was consistent with the literature, where the majority of PGE synthase activity elutes in the nuclear membrane fraction (21).

Spatial and temporal syntheses of PGE_2 in the uterus were assessed by immunohistochemistry. In the functionalis layer, PGE_2 synthesis was detected in epithelial, stromal, and endothelial cells (Fig. 2, b–f), with less apparent stromal cell immunoreactivity in late secretory tissue (Fig. 2e). As observed with PGE synthase expression, PGE_2 synthesis in the basalis region was minimal in the stromal compartment, whereas epithelial and endothelial immunoreactivities were unchanged. Full thickness endometrial biopsies clearly demonstrated the gradient in PGE_2 synthesis from strong im-

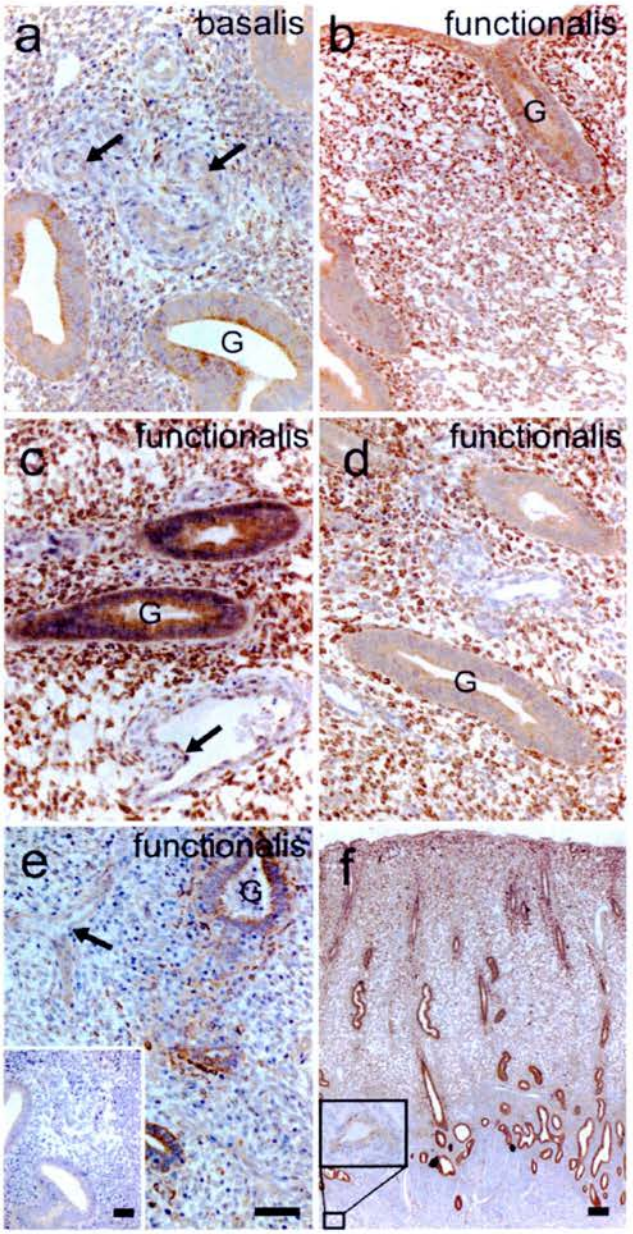

FIG. 1. Immunohistochemical localization of PGE synthase in the basalis and functionalis regions of the human endometrium. Lower PGE synthase immunoreactivity was detected in the stromal compartment of the basalis than in the functionalis region (a and b are the basalis and functionalis regions, respectively, of endometrial tissue collected during the midproliferative phase). In the functionalis region, PGE synthase immunoreactivity was detected at all stages of the menstrual cycle and was localized to glandular epithelial (G), stromal, and endothelial (denoted by *arrows*) cells (c, late proliferative; d, early secretory; e, late secretory). *Inset* in e, A section that was stained with preadsorbed PGE synthase antibody (negative control). f, Full thickness uterine tissue, collected during the late proliferative phase, demonstrating spatial changes in PGE synthase immunoreactivity between basalis and functionalis regions of the endometrium and the myometrium. *Inset* in f, Endothelial cell PGE synthase immunoreactivity within the myometrial compartment. *Scale bars*: e and *inset*, 100 μm; f, 500 μm.

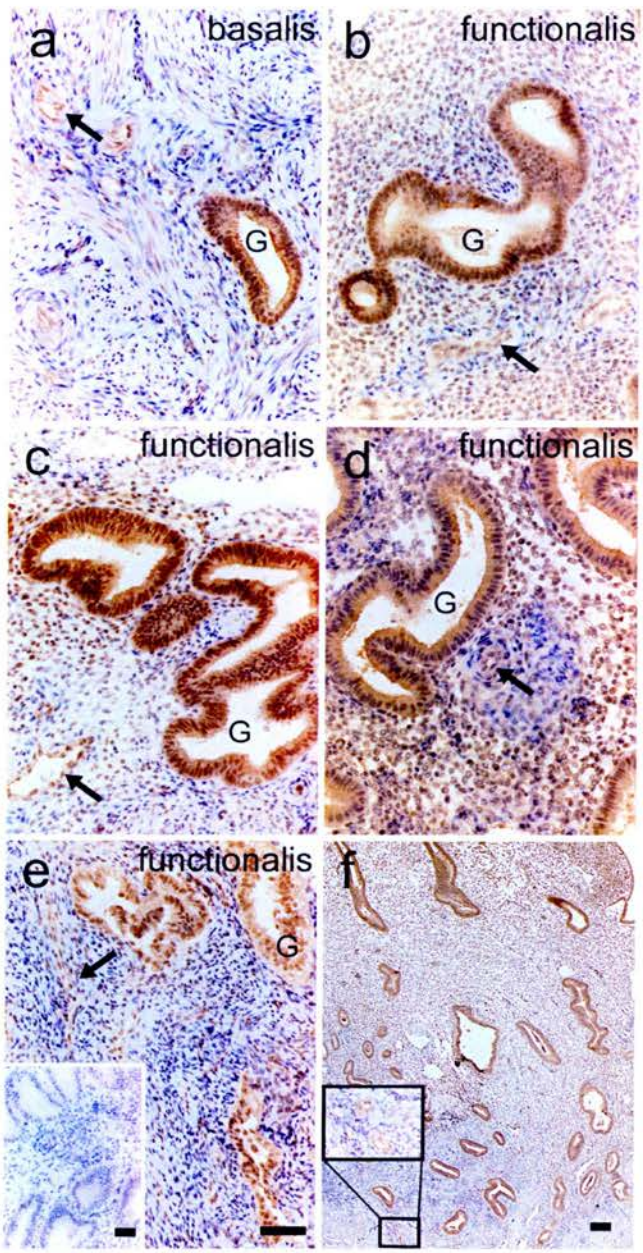

FIG. 2. Immunohistochemical localization of PGE_2 in the basalis and functionalis regions of the human endometrium. Lower PGE_2 immunoreactivity was detected in the stromal compartment of the basalis than in the functionalis region (a and b are basalis and functionalis regions, respectively, of endometrial tissue collected during the midproliferative phase). In the functionalis region, PGE_2 immunoreactivity was detected at all stages of the menstrual cycle and was localized to glandular epithelial (G), stromal, and endothelial (denoted by *arrows*) cells (c, late proliferative; d, early secretory; e, late secretory). *Inset* in e, Section that was stained with preadsorbed PGE_2 antibody (negative control). f, Full thickness uterine tissue, collected during the late proliferative phase, demonstrating spatial changes in PGE_2 immunoreactivity between basalis and functionalis regions of the endometrium and the myometrium. *Inset* in f, Endothelial cell PGE_2 immunoreactivity within the myometrial compartment. *Scale bars*: e and *inset*, 100 μm; f, 500 μm.

munoreactivity in cells of the functionalis layer to weak staining in the myometrium, where no fibroblast PGE_2 synthesis was observed. In the myometrium, similar to PGE synthase expression, PGE_2 synthesis was detected only in endothelial cells (Fig. 2F, *inset*).

Quantitation of EP2 receptor mRNA expression in endometrial biopsies demonstrated no significant change in expression across the menstrual cycle (Fig. 3a). EP4 receptor mRNA expression, in contrast, was significantly higher ($P < 0.05$) in late proliferative biopsies (0.162 ± 0.034; $n = 5$) than in early (0.075 ± 0.024; $n = 7$), mid (0.063 ± 0.014; $n = 6$), and late (0.07 ± 0.033; $n = 6$) secretory phase samples (Fig. 3b). It is noteworthy that receptor expression in these experiments was investigated in a heterogeneous population of uterine cells, and it is conceivable that variation in receptor expression across the menstrual cycle may be different in specific target cells.

The site of action of PGE_2 was investigated by conducting *in situ* hybridization for EP2 and EP4 receptors. The expression of both receptors was localized in the epithelial and vascular regions of human endometrium (Fig. 4, a–h). Furthermore, epithelial and vascular EP2 and EP4 receptor expression was present at all stages of the menstrual cycle in both the basalis and functionalis regions of the endometrium. Vascular EP2 and EP4 receptor mRNA expression was localized in both endothelial (Fig. 4, a and c, for EP2; Fig. 4, b and d, for EP4) and perivascular cells throughout the endometrium (Fig. 4g for EP2; Fig. 4h for EP4).

To investigate functional signaling of EP2/EP4 receptors,

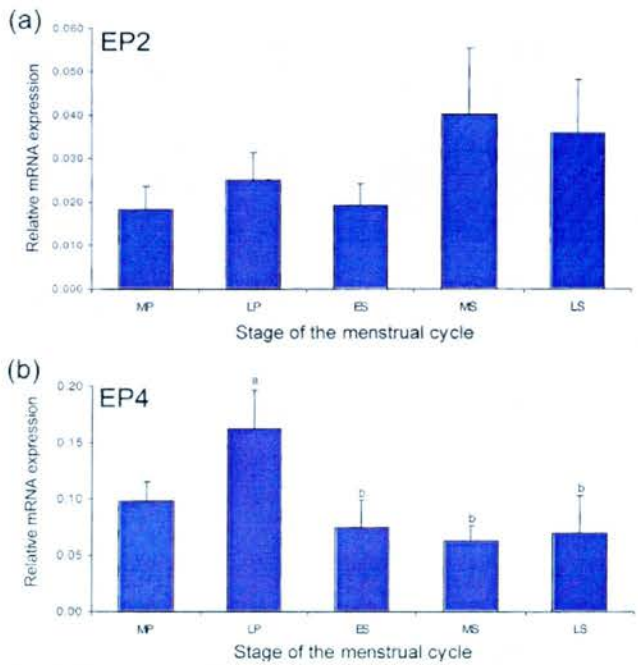

FIG. 3. Quantitative RT-PCR demonstrating relative expression of EP2 (a) and EP4 (b) receptors in midproliferative (MP; $n = 9$), late proliferative (LP; $n = 5$), early secretory (ES; $n = 7$), midsecretory (MS; $n = 6$), and late secretory (LS; $n = 6$) endometrial biopsies. Results are expressed as the mean ± SEM of relative mRNA expression levels. *Different letters* denote statistical difference ($P < 0.05$).

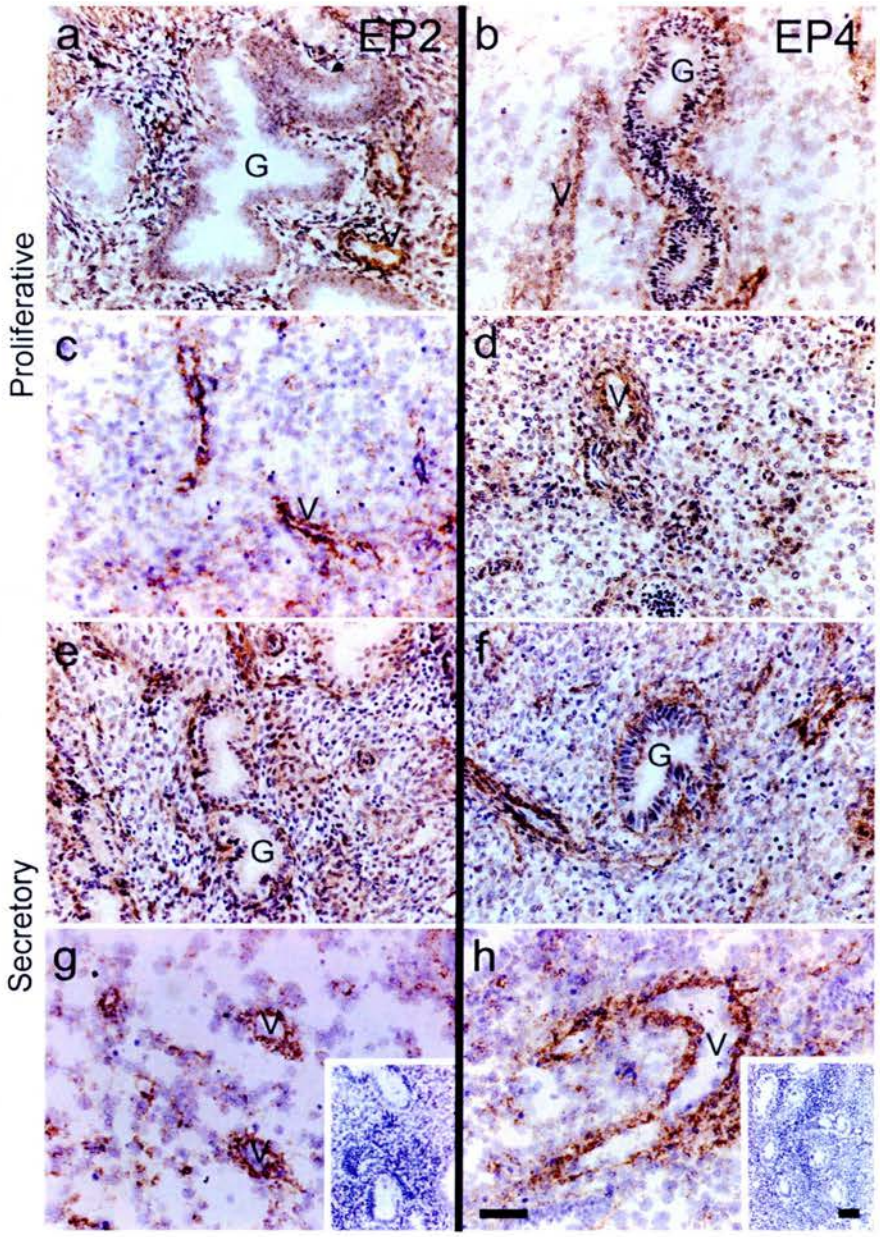

FIG. 4. *In situ* hybridization of EP2 (a, c, e, and g) and EP4 (b, d, f, and h) receptors in the human endometrium. EP2 and EP4 receptor expressions were localized to the glandular (G), stromal, and vascular (V) compartments of the human endometrium during the proliferative (a–d) and secretory (e–h) phases of the menstrual cycle. *Insets* in g and h, Sections treated with control riboprobe.

cAMP generation in response to exogenous treatment with PGE_2 was assessed in endometrial biopsy specimens collected across the menstrual cycle. cAMP generation in proliferative tissue was significantly higher (3.77 ± 0.85-fold; n = 6; $P < 0.05$) than that detected in early secretory (1.96 ± 0.28-fold; n = 6) or midsecretory (1.38 ± 0.23-fold; n = 6) tissue (Fig. 5).

Discussion

The data presented in this manuscript demonstrate the expression and localization of PGE synthase and its product, PGE_2, in stromal, epithelial, and endothelial cells of the human endometrium across the menstrual cycle. PGE synthase expression and PGE_2 synthesis were detected at all stages of the menstrual cycle, with apparent reduced expression/synthesis during the late secretory phase. This is in agreement with previous studies, which have localized COX expression in epithelial and perivascular cells of the human endometrium (14, 16–18). Moreover, culture studies have demonstrated that both epithelial and stromal cells can synthesize PGE_2 *in vitro* (22) and that the PGE_2 biosynthetic capacity of the endometrium is reduced in the late secretory phase (23). The sites of action of PGE_2 were elucidated using *in situ* hybridization studies for two receptor subtypes, namely EP2 and EP4. EP2 and EP4 receptor expression was localized in epithelial and vascular cells of human endometrium. These

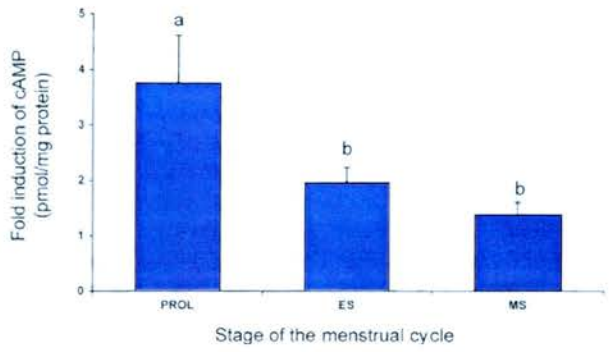

FIG. 5. Fold induction of cAMP production in endometrial biopsies collected from proliferative (PROL; n = 6), early secretory (ES; n = 6), and midsecretory (MS; n = 6) stages of the menstrual cycle after stimulation with 300 nM PGE_2. Results are expressed as the mean ± SEM fold cAMP induction. *Different letters* denote statistical difference ($P < 0.05$).

data indicate that PGE_2 acts in an autocrine/paracrine manner within multiple cellular compartments of the human endometrium.

In premenopausal women, the human endometrium undergoes phases of proliferation and apoptosis during successive menstrual cycles. These phases are observed predominantly in the functionalis layer of the endometrium, which is shed at menstruation before regenerating during the proliferative phase of the subsequent menstrual cycle. Interestingly, stromal expression of PGE synthase and synthesis of PGE_2 are predominantly localized in the functionalis layer of the endometrium and are minimal in the basalis and myometrial regions. The exact role of PGE_2 in the human endometrium is not fully elucidated, but previous studies suggest a crucial role for PGE_2 in cellular mitogenesis and survival. In colon epithelial cells, overexpression of COX-2 and enhanced synthesis of PGE_2 have been shown to promote the proliferation and survival of cells through inhibition of apoptosis (9, 10). The latter effect is mediated via up-regulated expression of antiapoptotic genes such as *bcl-2* (10). It is plausible that in the human endometrium, PGE_2 may be activating similar mechanisms that promote glandular epithelial cell proliferation and/or survival. This is supported by recent data confirming higher expression of *bcl-2* in the proliferative compared with secretory phase endometrium and its localization to glandular epithelial cells (24–26). PGE_2 function in glandular epithelial cells may also be associated with the regulation of uterine angiogenesis. Overexpression of COX-2 and increased production of PGE_2 in epithelial cells have been associated with the expression of angiogenic factors, such as vascular endothelial growth factor, which, in turn, act in a paracrine manner to induce endothelial cell migration and microvascular tube formation (11). In the human endometrium, vascular endothelial growth factor expression is localized to the glandular epithelial cells throughout the menstrual cycle (27, 28), and PGE_2 has been shown previously to up-regulate the expression of vascular endothelial growth factor in a number of different cell types via cAMP (29–32).

Expression of EP2/EP4 receptors in perivascular and endothelial cells suggests a possible role for PGE_2 in vascular function and angiogenesis in the human endometrium. PGE_2 induces vasodilatation via perivascular EP2/EP4 receptors (1), whereas activation of EP receptors on endothelial cells may regulate angiogenesis. Endometrial angiogenesis takes place throughout the menstrual cycle, as measured by endothelial cell proliferation, with a significantly elevated endothelial cell proliferative index in the functionalis compared with the basalis phase (33). Recently, COX-2 and PGE_2 have been linked directly with endothelial cell function and angiogenesis (12). Treatment of endothelial cells with selective COX-2 inhibitors has been shown to reduce microvascular tube formation, and this effect is partially reversed by cotreatment with PGE_2 (13). Hence, it is feasible that *in vivo* angiogenesis in the endometrium may be regulated by PGE_2 via an epithelial-endothelial and an endothelial-endothelial cell interaction. This is supported by the data presented in this study, which localized the site of expression of PGE synthase, PGE_2, and EP2/EP4 receptors to epithelial and endothelial cells of the endometrium.

Measuring cAMP generation in human endometrium after treatment with PGE_2 assessed functional signaling of EP2/EP4 receptors. A significantly higher cAMP generation was observed in proliferative compared with secretory endometrium. This enhanced signaling is associated with greater expression of EP4 receptors in the proliferative phase. The increased cAMP generation observed during the proliferative phase may mediate the mitogenic effect of PGE_2 on glandular epithelial and endothelial cells. PGE_2 has been shown previously to induce epithelial cell proliferation via the cAMP/protein kinase A pathway (34).

Further evidence of a role for PGE_2 in uterine vascular function can be derived from studies of menorrhagia. Nonsteroidal antiinflammatory drugs, which inhibit PG synthesis, are the treatment of choice to alleviate excessive blood loss in women reporting menorrhagia (35). Interestingly, increased binding of PGE_2, suggesting increased receptor expression, has been observed in the uteri of patients reported to suffer from menorrhagia (36). Moreover, treatment with fenamates (nonsteroidal antiinflammatory drugs) to reduce menstrual blood loss also reduces PGE_2-binding sites within the uterus (37). Together these data indicate an *in vivo* relationship between EP receptor expression and menstrual blood loss.

In conclusion, the data presented herein confirm the expression of PGE synthase, PGE_2, and functional EP2 and EP4 receptors in epithelial and endothelial cells of human endometrium. In addition, we observed increased EP4 receptor and PGE_2-induced cAMP production in proliferative phase tissue. We hypothesize that PGE_2 is an integral modulator of cell proliferation and/or differentiation within the endometrium and acts as an autocrine/paracrine factor between epithelial and endothelial cells.

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Cyclooxygenase-2 Overexpression Inhibits Cathepsin D-Mediated Cleavage of Plasminogen to the Potent Antiangiogenic Factor Angiostatin

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Overexpression of cyclooxygenase (COX)-2 and enhanced synthesis of prostaglandin E_2 (PGE_2) have been implicated in human endometrial pathologies. To investigate the molecular role of COX-2, the Ishikawa human endometrial epithelial cell line was stably transfected with the pIRES2 vector containing COX-2 cDNA in either the sense or antisense directions. PGE_2 concentrations were significantly elevated in the cells transfected with the COX-2 sense compared with wild-type cells or cells transfected with the antisense cDNA ($P < 0.01$). Elevated PGE_2 synthesis was associated with enhanced expression and signaling of PGE_2 receptors (EP). cDNA array analysis revealed differential expression of cathepsin D between the COX-2 sense and antisense cells. Cathepsin D RNA and protein expression was 6.7- and 2.1-fold lower in the COX-2 sense compared with COX-2 antisense cells respectively. Cathepsin D is known to cleave plasminogen to the potent antiangiogenic factor angiostatin. To investigate differential angiostatin generation, conditioned media from COX-2 sense, COX-2 antisense and wild-type cells were incubated with plasminogen and subsequently subjected to Western blot analysis. In comparison to wild-type cells, the cleavage of plasminogen to angiostatin was abolished when incubated in COX-2 sense cells conditioned media and elevated when incubated in COX-2 antisense cells conditioned media. Coincubation of plasminogen with the cathepsin D inhibitor pepstatin A inhibited the cleavage of plasminogen to angiostatin in the COX-2 antisense conditioned media. These data demonstrate that COX-2 exerts a negative feedback on the expression of cathepsin D. This in turn reduces the generation of the antiangiogenic factor angiostatin, hence promoting a proangiogenic environment. (*Endocrinology* 144: 5322–5328, 2003)

TWO PREDOMINANT ISOFORMS of the cyclooxygenase (COX) enzymes have been identified (COX-1 and COX-2). COX-1 is constitutively expressed in many cell types and has been shown recently to be inducible in certain cancers (1–4). COX-2 is the readily inducible form of the enzyme and is commonly associated with several pathological conditions including tumorigenesis (5, 6). The COX enzymes catalyze the rate-limiting step in the biosynthetic pathway of prostanoids. There are five endogenous prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane A2 (7). Arachidonic acid, once released from the membrane phospholipids is converted to the prostanoid intermediate PGH_2 by the COX enzymes. PGH_2 acts as a substrate for synthases specific to each prostanoid such as PGE synthase (PGES) for PGE_2 (8, 9). Once synthesized, PGE_2 elicits its effects via its seven *trans*-membrane G protein-coupled receptors, of which four have been identified (EP1, EP2, EP3, and EP4). These receptors signal via alternate and in some cases opposing signaling pathways (7, 10). EP1 receptor activation leads to elevated inositol-3-phosphate and Ca^{2+} levels, activation of both EP2 and EP4 results in increased intracellular cAMP levels and depending on the splice variant, EP3 activation either decreases or increases cAMP levels (7).

Numerous studies have demonstrated that overexpression of COX-2 in epithelial cells is associated with enhanced production of angiogenic factors (11, 12). These factors act in a paracrine manner to promote endothelial cell migration and microvascular tube formation (12). In the female reproductive tract, a role for COX enzymes and PGE_2 in normal and pathological angiogenesis has been proposed. In the human endometrium expression/synthesis and signaling of COX-2, PGE_2, and EP receptors colocalize in glandular epithelial and endothelial cells of the normal and neoplastic endometrium (13–19). Moreover, overexpression of COX enzymes in epithelial cells of the reproductive tract has been shown to promote the expression of various angiogenic factors (4).

A role for COX enzymes has also been proposed in benign pathologies of the endometrium such as endometriosis, dysmenorrhea, and heavy menses (20–26). Furthermore, several studies have associated heavy menses with abnormalities in vasodilatatory prostanoid production such as PGE_2 from the uterus (20–22). PGE_2 synthesis and PGE_2 binding sites in uterine tissues are greater in women diagnosed with heavy menses compared with women with normal blood loss (18, 21, 27–29). The elevated prostanoids detected in menstrual flow of patients with heavy menses has lead to the administration of COX enzyme inhibitors as a means of therapy (30). COX enzyme inhibitors such as ibuprofen have been shown to reduce menstrual blood loss (31). This suggests that the degree or duration of menstrual bleeding in women diagnosed with menorrhagia is augmented following elevation of vasodilatatory factors by COX enzyme products.

This study was designed to investigate the potential role of COX-2 in regulating endometrial epithelial cell function. The specific aims of the study were to examine the effect of

Abbreviations: COX, Cyclooxygenase; EP, PGE_2 receptor; PGE, prostaglandin E; PGI_2, prostacyclin; PGES, PGE synthesis.

COX-2 overexpression in an endometrial epithelial cell line (Ishikawa) on PGE_2 secretion, EP receptor expression and signaling and to identify genes regulated by COX-2 that may be associated with endometrial function and angiogenesis.

Materials and Methods

Cell culture

Ishikawa wild-type, COX-2 sense, and COX-2 antisense cells were routinely maintained in DMEM nutrient mixture F-12 with glutamax-1 and pyridoxine, supplemented with 10% fetal bovine serum and 1% antibiotics (500 U/ml penicillin and 500 μg/ml streptomycin) at 37 C and 5% CO_2 (vol/vol). In addition, COX-2 sense and COX-2 antisense cells were maintained in media containing 800 μg/ml G418 (Calbiochem, Nottingham, UK).

Transfection of cells

The pBS(SK-)PSHI containing the full-length COX-2 cDNA (kindly supplied by Dr. Stephen Prescott, University of Utah, Salt Lake City, UT) was used as the template plasmid. The COX-2 cDNA was excised from the template plasmid and ligated at the *Eco*R1 site of the pIRES2 vector (CLONTECH, Hampshire, UK). The orientation of the COX-2 cDNA insert was determined by dideoxy DNA sequencing using sequence specific primers for pIRES2. Wild-type Ishikawa cells were plated in a 12-well plate at a density of 1.2×10^5 cells per well and left to attach overnight. The following day, the pIRES2 vector containing the COX-2 cDNA in either the sense or antisense directions was transfected into the Ishikawa endometrial epithelial cell line using pfx-5 (Invitrogen, Paisley, Scotland, UK) diluted in Optimem (Life Technologies, Inc., Paisley, Scotland, UK). Following a 4-h incubation at 37C, 5% CO_2 (vol/vol), the transfection mixture was replaced with fresh complete media. The transfected cells were allowed to grow for 24 h and then seeded with wild-type cells and selected using G418 (Calbiochem; at a concentration of 800 μg/ml). A total of 120 colonies with the COX-2 sense cDNA and 60 colonies with the COX-2 antisense cDNA were picked using cloning rings. The clones were screened for COX-2 protein expression using Western blot analysis. Initial experiments were performed on four COX-2 sense and two COX-2 antisense clones. All of the COX-2 sense clones generated significantly higher PGE_2 into the culture media compared with COX-2 antisense and wild type. The data presented in the manuscript and all further investigations were performed using the COX-2 sense 72 clone and COX-2 antisense 15 clone as these generated the highest and lowest levels of PGE_2, respectively.

Protein extraction and Western blot analysis

Ishikawa wild-type, COX-2 sense, and COX-2 antisense cells were seeded at a density of 7.5×10^5 cells in six-well plates, allowed to attach for 24 h in complete media and then cultured in serum free media overnight (n = 4 independent experiments). Subsequently, cells were lysed on the plates for 20 min with the lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.6% Nonidet P-40, 1 mM Na_3VO_4, 10% glycerol, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride]. The lysates were clarified by centrifugation and the supernatants collected. Protein concentration was determined by the modified Lowry method (Bio-Rad D2 Protein Assay kit, Bio-Rad Laboratories, Hemel Hempstead, UK). A total of 20-μg protein for cathepsin D expression and 40 μg protein for COX-2, COX-1, and β-actin, expression were denatured and subjected to SDS-PAGE on 4–12% Tris-glycine gels (Invitrogen). The proteins were transferred onto polyvinylidene difluoride membrane and blocked for 1 h in TBS-Tween [50 mM Tris-HCl, 150 mM NaCl, and 0.05% (vol/vol) Tween 20 containing 5% skimmed milk powder]. The membranes were probed with one of the following antibodies: COX-2 (sc-1745; at dilution of 1:1000), COX-1 (sc-1752; at dilution of 1:500), β-actin (sc-1616; at dilution of 1:1000), cathepsin D (sc-6486; at dilution of 1:2000) or plasminogen (sc-15034; at dilution of 1:500) overnight followed by rabbit antigoat conjugated to alkaline phosphatase secondary antibody at a dilution of 1:30,000 (Sigma, Poole, UK). The specificity of some of the antibodies has been confirmed previously in our laboratory by preadsorbtion of the antibodies to the respective blocking peptides (4). All the primary antibodies were purchased from Santa Cruz Biotechnology (Autogenbioiclear, Whiltshire, UK). The membranes were developed and revealed by PhosphorImager analysis using the ECF chemifluorescence system according to the manufacturer's instructions (Amersham Biosciences UK Ltd., Little Chalfont, UK). The molecular weights of the proteins were determined by comparing mobility on the gel with a molecular weight standard (Invitrogen). Protein bands were semiquantified by densitometry using STORM 860 system (Molecular Dynamics, Amersham Biosciences, Buckinghamshire, UK). Relative expression of cathepsin D protein was calculated by normalizing with β-actin and expressed as mean ± SEM.

PGE_2 assay

Ishikawa wild-type, COX-2 sense, and COX-2 antisense cells were seeded in six-well plates at a cell density of 3.5×10^5 cells per well, allowed to attach for 24 h in complete media and then cultured in serum free media overnight (n = 4 independent experiments). The cells were incubated for a further 48 h in serum-free media containing 5 μg/ml arachidonic acid in the presence or absence of 10 μM NS398 (Calbiochem, Nottingham, UK). PGE_2 secretion in the culture media was assayed using an ELISA as described by Denison *et al.* (32). The data are presented as mean ± SEM. The intra- and interassay coefficients of variation were 7.8% and 15.0%, respectively, with an assay detection limit of 10 pg/ml.

Taqman quantitative RT-PCR

To determine the effect of COX-2 on EP receptor expression, wild-type, COX-2 sense, and COX-2 antisense cells were seeded at a density of 5×10^5 in six-well plates, allowed to attach for 24 h in complete media and then cultured in serum-free media overnight (n = 3 independent experiments). These experiments were conducted in the absence of NS398 as recent data suggest that this inhibitor up-regulates the expression of EP receptors (33). Thereafter RNA was extracted using Tri Reagent (Sigma) following the manufacturer's instructions. RNA samples were quantified and reverse transcribed using 5.5 mM $MgCl_2$, 0.5 mM each deoxynucleotide triphosphates, 2.5 μM random hexamers, 0.4 U/ml ribonuclease inhibitor, and 1.25 U/ml Multiscribe reverse transcriptase (all from PE Applied Biosystems, Warrington, UK). A total of 400 ng template RNA was added to RT mix and incubated for 60 min at 25 C, 45 min at 48 C, and 5 min at 95 C. The PCR mix consisted of 1× Universal PCR Mastermix, forward and reverse primers for either EP1, EP2, EP3, or EP4 (300 nM) and EP1, EP2, EP3, or EP4 probe (200 nM) and ribosomal 18S forward primer, reverse primer and probe (50 nM; all from PE Applied Biosystems, Warrington, UK). For PCR, 48 μl of PCR mix was mixed with 2 μl cDNA and subsequently a volume of 24 μl of this mixture was placed in duplicate into wells on a PCR plate along with a no template control. The wells were sealed using optical lids and the PCR was carried out using an ABI Prism 7700 (PE Applied Biosystems). The primers for the EP receptors and 18S were designed using PRIMER express software (PE Applied Biosystems) and the sequences are presented in Table 1. 18S rRNA was used as an internal standard to normalize the samples for RNA loading. Results were expressed relative to a positive standard (cDNA obtained from a single sample of endometrial tissue) run in each PCR. Relative EP receptor expression was calculated by dividing EP receptor expression in the COX-2 sense and COX-2 antisense cells by expression detected in wild type. Data are presented as mean ± SEM.

cAMP turnover

Ishiwawa wild-type, COX-2 sense, and COX-2 antisense cells were seeded in six-well plates at a cell density of 2×10^5 cells per well, allowed to attach for 24 h in complete media and then cultured in serum free media overnight (n = 4 independent experiments). The media was then replaced with fresh serum free media containing 1 mM 1-methyl-3-isobutylxanthine (Sigma) for 1.5 h. Cells were stimulated with 0 or 100 nM PGE_2 for 10 min. The media was removed and the cells were lysed using 0.1 M HCl. cAMP generation was quantified using a cAMP ELISA kit (Biomol, Affiniti, Exeter, UK) according to the manufacturer's instructions and normalized to protein concentrations of the homogenate. Protein concentrations were determined using protein assay kits (Bio-Rad Laboratories). The data are presented as the mean (±SEM), fold

TABLE 1. Oligonucleotide sequences for the various EP receptor primers and probes

Gene		Primers and probe
EP1	Forward primer	5′-AGA TGG TGG GCC AGC TTG T-3′
	Reverse primer	5′-GCC ACC AAC ACC AGC ATT G-3′
	FAM linked probe	5′-CAG CAG ATG CAC GAC ACC ACC ATG-3′
EP2	Forward primer	5′-GAC CGC TTA CCT GCA GCT GTA-3′
	Reverse primer	5′-TGA AGT TGC AGG CGA GCA-3′
	FAM linked probe	5′-CCA CCC TGC TGC TGC TTC TCA TTG TCT-3′
EP3	Forward primer	5′-GAC GGC CAT TCA GCT TAT GG-3′
	Reverse primer	5′-TTG AAG ATC ATT TTC AAC ATC ATT ATC A-3′
	FAM linked probe	5′-CTG TCG GTC TGC TGG TCT CCG CTC-3′
EP4	Forward primer	5′-ACG CCG CCT ACT CCT ACA TG-3′
	Reverse primer	5′-AGA GGA CGG TGG CGA GAA T-3′
	FAM linked probe	5′-ACG CGG GCT TCA GCT CCT TCC T-3′
18S	Forward primer	5′-CGG CTA CCA CAT CCA AGG AA-3′
	Reverse primer	5′-GCT GGA ATT ACC GCG GCT-3′
	VIC linked probe	5′-TGC TGG CAC CAG ACT TGC CCT C-3′

induction of cAMP in COX-2 sense and antisense cells after treatment with PGE_2 where fold induction was calculated relative to the wild-type samples. The intra- and interassay coefficients of variation were 8.3% and 11.6%, respectively.

cDNA array analysis

Differential gene expression in the COX-2 sense and antisense cells was assessed using cDNA array analysis. COX-2 sense and COX-2 antisense cells were grown to approximately 70% confluency in complete media. The cells were cultured in serum free media overnight and then harvested by trypsinization for 5 min. The cells were resuspended in PBS, pelleted by centrifugation, snap frozen on dry ice, and stored at −70 C. The cell pellets were sent to CLONTECH Laboratories Inc. (Palo Alto, CA) for custom cDNA array analysis using the Atlas plastic human 8K gene Microarray service. This array includes a list of 8000 genes that are involved in diverse molecular and cellular functions (for further information visit www.clontech.com). The results were analyzed using AtlasImage software and expressed as a comparison between the COX-2 sense and COX-2 antisense cells.

Determination of angiostatin generation

Ishikawa wild-type, COX-2 sense, and COX-2 antisense cells were seeded in six-well plates at a density of 3.5×10^5, allowed to attach for 24 h in complete media and then cultured in serum-free media overnight ($n = 3$ independent experiments). Following culture, the media was collected, spun at $1000 \times g$ for 5 min to pellet cell debris, and the supernatant was aspirated. To investigate the differential cleavage of plasminogen to angiostatin by the three cell lines, aliquots of cell media (100 μl) were incubated with 25 μg /ml plasminogen at 37 C for 0, 4, 8, or 24 h. To investigate whether cathepsin D mediates the cleavage of plasminogen to angiostatin, 100 μl of media from COX-2 antisense cells were incubated in the presence or absence of 1 μM pepstatin A (Sigma) for 8 h. Subsequently, a total of 20 μl of each reaction was denatured and subjected to SDS-PAGE on 4–12% Tris-glycine gels (Invitrogen). The generation of angiostatin from plasminogen was assessed by Western blot analysis and probed with a plasminogen antibody as described above

Statistical analyses

The data in this study were analyzed by ANOVA using StatView 5.0 software (Abacus Concepts, Berkeley, CA). Statistical significance was taken as $P < 0.05$.

Results

Stable transfection of the Ishikawa human endometrial epithelial cells with the pIRES2 vector containing COX-2 cDNA in the sense direction resulted in the overexpression of COX-2 protein. Western blot analysis revealed strong COX-2 protein expression detected as 72-kDa band in the COX-2 sense cells compared with the COX-2 antisense and wild-type cells (Fig. 1A). Basal levels of COX-2 were detected in the wild-type Ishikawa cells that was abolished by transfection with COX-2 antisense cDNA. (Fig. 1A). Stable transfection of the COX-2 cDNA in either the sense or antisense direction had no effect on COX-1 protein expression (Fig. 1B); no differences were detected in COX-1 protein levels between the wild-type, COX-2 sense, or COX-2 antisense cell lines.

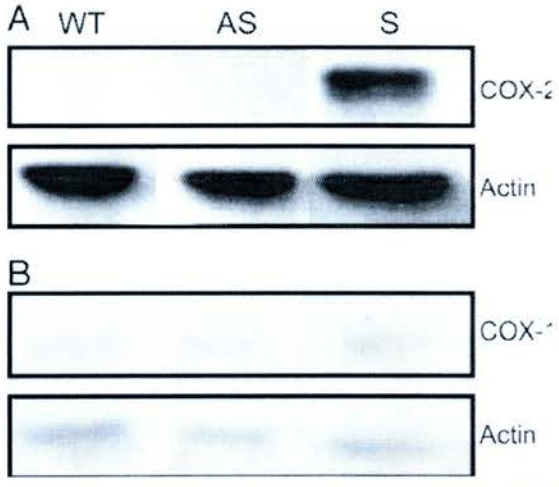

FIG. 1. Western blot analysis of 40 μg protein from wild-type Ishikawa cells (WT) and Ishikawa cells stably transfected with COX-2 cDNA in either the sense (S) or antisense (AS) directions. A, Specific bands for COX-2 and β-actin proteins were detected at approximately 72 and 46 kDa, respectively. B, COX-1 and β-actin proteins were detected by specific bands at 71 and 46 kDa, respectively.

To confirm functionality of the transfected cDNA, PGE_2 synthesis was measured by ELISA in COX-2 sense, COX-2 antisense and wild-type cells (Fig. 2). PGE_2 secretion was significantly increased ($P < 0.01$) in the culture media of the COX-2 sense cells (2391.25 ± 311.09 pg/ml) compared with wild-type and COX-2 antisense cells (359 ± 71.54 and 78.96 ± 50.9 pg/ml, respectively). Coculture of the cells with the specific COX-2 enzyme inhibitor NS398 reduced the in-

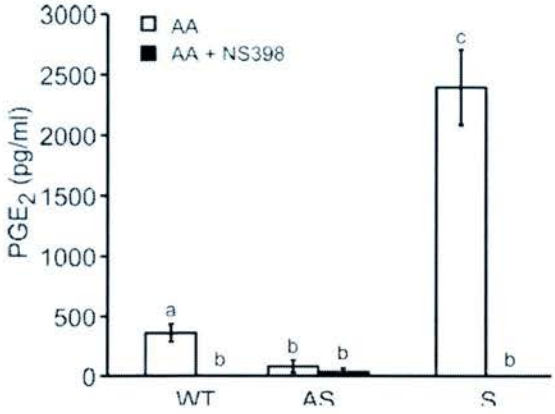

FIG. 2. The functionality of the transfected COX-2 was assessed by ELISA to measure PGE_2 secretion by wild-type (WT), COX-2 antisense (AS), and COX-2 sense (S) cells into culture media following incubation with 5 μg arachidonic acid (AA) for 48 h in the presence or absence 10 μM of the COX-2 enzyme inhibitor NS398. The data are mean ± SEM of n = 4 experiments. *Different letters* denote statistical significance ($P < 0.05$).

creased secretion of PGE_2 in the wild-type, COX-2 sense, and COX-2 antisense cells to almost undetectable levels. To investigate the effect of COX-2 overexpression on EP receptors, real time quantitative RT PCR was performed to quantify the expression of EP1, EP2, EP3, and EP4 receptors (Fig. 3A). Relative expression of EP2 and EP3 mRNA (compared with expression levels detected in wild-type cells) was significantly higher ($P < 0.05$) in the COX-2 sense cells compared with COX-2 antisense cells (EP2 receptor: 3.73 ± 0.43 *vs.* 1.19 ± 0.39 for COX-2 sense and antisense cells, respectively; EP3 receptor: 2.35 ± 0.3 *vs.* 1.03 ± 0.105 for COX-2 sense and antisense cells, respectively). EP1 mRNA was not detectable in any of the cell lines and EP4 receptor expression showed no significant differences between the COX-2 sense and antisense cells. Functionality of the EP receptors was investigated by measuring the generation of cAMP in COX-2 sense, COX-2 antisense and wild-type cells in response to treatment with 100 nM PGE_2 for 10 min (Fig. 3B). Fold induction of cAMP relative to that measured in wild-type cells was significantly higher ($P < 0.05$) in the COX-2 sense cells compared with the COX-2 antisense cells (2.57 ± 0.16 *vs.* 0.88 ± 0.33 for COX-2 sense and antisense cells, respectively).

Differential gene expression between COX-2 sense and COX-2 antisense cells was investigated using RNA extracted from the two cell lines and the CLONTECH Atlas human 8K microarrays. A number of genes demonstrated differential expression between the two cell lines. Of the genes differentially expressed, one of the greatest differences observed was cathepsin D. Cathepsin D mRNA expression demonstrated a 6.7-fold difference between the COX-2 sense and the COX-2 antisense cells; cathepsin D RNA expression was reduced in the COX-2 sense compared with the COX-2 antisense cells (Fig. 4A). Western blot analysis conducted using proteins extracted from the wild-type, COX-2 sense, and COX-2 antisense cell lines confirmed lower cathepsin D protein expression in the COX-2 sense and elevated expression in the COX-2 antisense cells compared with the wild-type cells. Cathepsin D protein expression in the COX-2 sense and COX-2 antisense cell lines was 0.8 ± 0.13 and

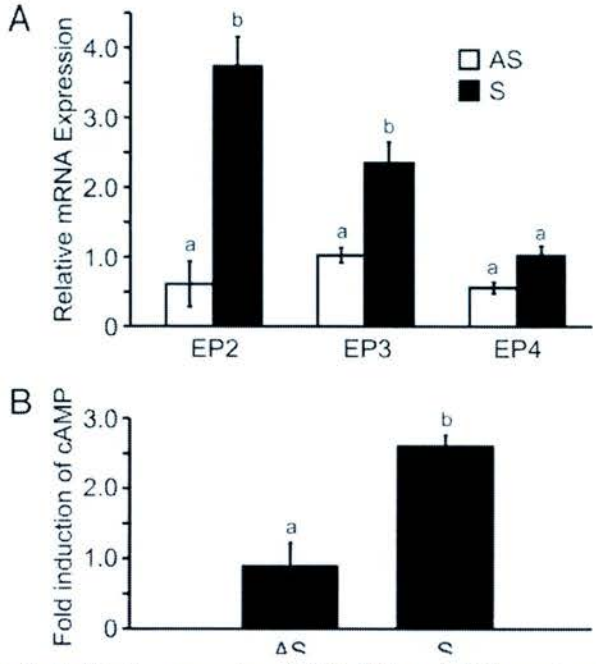

FIG. 3. A, Relative expression of EP2, EP3, and EP4 receptors in Ishikawa cells stably overexpressing COX-2 in either the sense (S) or antisense (AS) directions. Relative expression was determined by dividing expression detected in COX-2 sense and antisense cells by expression detected in wild-type cells. The data are mean ± SEM of n = 3 experiments. *Different letters* for each of the receptors denote statistical significance ($P < 0.05$). B, ELISA for cAMP generation. Fold induction of cAMP generation in COX-2 antisense (AS) and COX-2 sense (S) cells following treatment with 100 nM PGE_2 for 10 min. Fold induction is calculated by dividing cAMP generation in COX-2 sense and antisense cells by cAMP generation in wild-type cells. The data are mean ± SEM of n = 4 experiments. *Different letters* denote statistical significance ($P < 0.05$).

1.68 ± 0.39-fold relative to wild-type cells. The difference in cathepsin D expression was 2.16 ± 0.22-fold lower in the COX-2 sense compared with COX-2 antisense cells $P < 0.05$ (Fig. 4, B and C).

We investigated the potential effect of differential cathepsin D expression in the three cell lines, on cleavage of plasminogen to angiostatin. Plasminogen was cultured in the media collected from COX-2 sense, COX-2 antisense, and wild-type cells for 0, 4, 8, and 24 h. Using antiplasminogen antibody and Western blot analysis, angiostatin bands were detected at 36 and 32 kDa, which are within the reported range (34, 35). Angiostatin production was elevated following culture of plasminogen in media collected from COX-2 antisense cells compared with wild-type cells. However, angiostatin production was abolished when plasminogen was incubated in the culture media collected from COX-2 sense cells (Fig. 5). To confirm that the cleavage of plasminogen to angiostatin is mediated by cathepsin D, the incubation experiments were repeated by incubating plasminogen in COX-2 antisense conditioned media in the presence of the cathepsin D inhibitor pepstatin A. Coincubation of plasminogen and cathepsin D in the conditioned media from COX-2 antisense cells for 8 h resulted in inhibition of angiostatin production (Fig. 6).

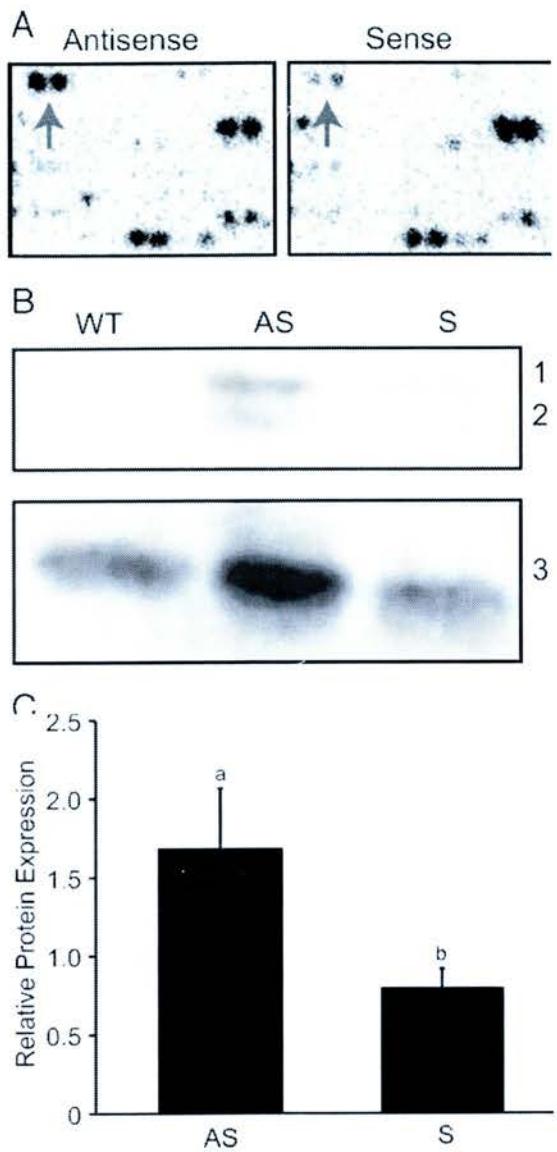

FIG. 4. A, Atlas plastic 8K Human cDNA array image following hybridysation with cDNA from untreated COX-2 antisense and COX-2 sense cells. *Arrows* correspond to cathepsin D position. B, Western blot analysis of 20 μg protein isolated from untreated Ishikawa wild-type (WT), COX-2 antisense (AS), and COX-2 sense (S) cells. The blot was probed with cathepsin D antibody that detected procathepsin D (1), pseudocathepsin D (2) and cathepsin D (3) protein expression at 52 kDa, 51 kDa, and 32 kDa, respectively. C, Relative expression of cathepsin D in COX-2 antisense and COX-2 sense cells normalized for β-actin and expressed relative to expression detected in wild-type cells. Bands were semiquantified as outlined in *Materials and Methods* and presented as mean ± SEM relative expression of n = 4 experiments. *Different letters* denote statistical significance ($P < 0.05$).

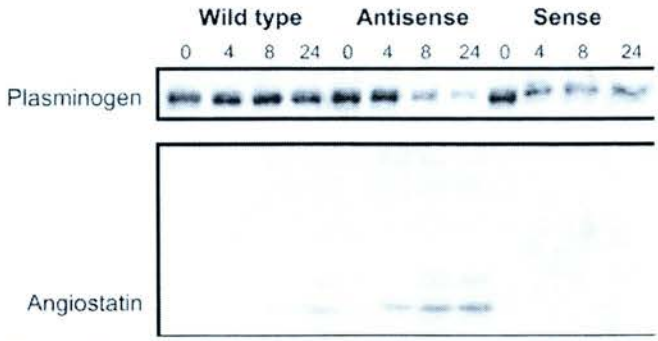

FIG. 5. Generation of angiostatin following incubation of 25 μg /ml plasminogen for 0, 4, 8, and 24 h in serum-free conditioned media collected from Ishikawa wild-type, COX-2 antisense, and COX-2 sense cells. Angiostatin production was detected by Western blot analysis using 20 μl of media. Plasminogen and angiostatin were detected at expected molecular masses of 97 and 36/32 kDa, respectively.

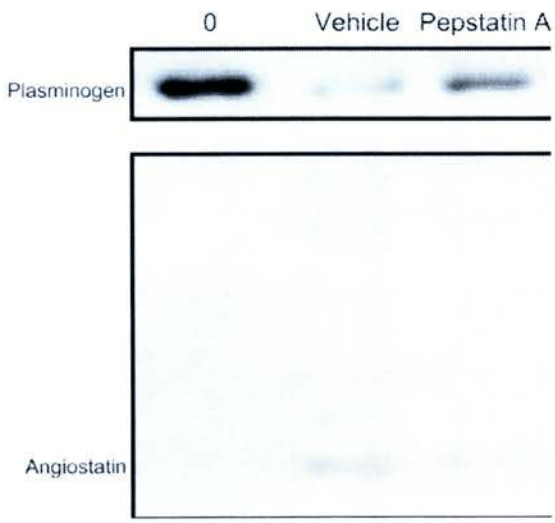

FIG. 6. Inhibition of angiostatin generation by pepstatin A following incubation of 25 μg/ml plasminogen in media collected from COX-2 antisense cells in the presence or absence of 1 μM pepstatin A for 8 h. Angiostatin production was detected by Western blot analysis using 20 μl of media. Plasminogen and angiostatin were detected at expected molecular masses of 97 and 36/32 kDa, respectively.

Discussion

In this study, we describe the establishment of an endometrial epithelial cell line stably overexpressing COX-2. COX-2 protein expression was higher in the sense clone compared with the antisense and wild type. Overexpression of COX-2 in the Ishikawa endometrial epithelial cells was associated with elevated secretion of PGE_2 that was abolished by the COX-2 selective inhibitor NS398. This is in agreement with previous studies that have demonstrated increased prostanoid generation in response to the overexpression of COX-2 (12). Similar expression of COX-1 protein was detected in all three cell lines, suggesting that COX-1 expression is not regulated by COX-2. Treatment of the cells with NS398 reduced PGE_2 secretion in wild-type, COX-2 sense, and COX-2 antisense cells to almost undetectable levels. This suggests that PGE_2 secretion in the wild-type, COX-2 sense, and COX-2 antisense cell lines is predominantly a result of COX-2 activity. COX-2 overexpression was also associated with increased EP2/EP3 receptor expression and signaling with no effect on EP1 and EP4 receptor expression. The elevation in EP receptor expression in the COX-2 sense cells suggests that COX enzyme products act in an auto-

crine/paracrine manner to up-regulate EP receptor expression. The COX-2 sense cells displayed also significantly higher fold induction of cAMP relative to the COX-2 antisense cells. The EP2 receptor is associated with Gs coupled signaling and results in enhanced cAMP generation (7), whereas a number of splice variants have been described for the EP3 receptor and these have been shown to be linked to either the Gs- or Gi-coupled signaling (7). The net increase in cAMP in response to exogenous PGE_2 in the COX-2 sense cells suggests an overall balance toward Gs coupled signaling in response to exogenous PGE_2 is favored. Hence, these data confirm that prostanoids such as PGE_2 secreted in response to COX-2 regulate the expression of their receptors and regulate gene expression in an autocrine/paracrine manner.

To identify differential gene expression between the COX-2 sense and the COX-2 antisense cells, we employed cDNA array analysis. We demonstrated reduced cathepsin D mRNA and protein expression in the COX-2 sense cells compared with wild-type and antisense cells. In the human endometrium, cathepsin D expression has been localized to glandular and stromal cells, and glandular expression has been shown to be highest during the secretory phase of the menstrual cycle (36). The elevated expression of cathepsin D is thought to be positively regulated by progesterone. However, cathepsin D expression remains elevated in the mid to late secretory phase despite a reduction in progesterone receptor activity. This has prompted the suggestion that cathepsin D expression is also under the control of other factors (37). Our data demonstrating that cathepsin D expression is inhibited via a COX-2 mediated action suggests COX-2 may be one of those factors. COX-2 expression in the human endometrium is highest during the late secretory and proliferative phases of the menstrual cycle (14) when cathepsin D levels have been reported to be at their lowest. Interestingly, elevated COX-2 and reduced cathepsin D have independently been associated with a poor prognosis in reproductive tract carcinoma (38–40). Moreover, COX-2 has been shown to be induced in endometrial adenocarcinomas (15–17). Hence it is plausible to suggest that increased COX-2 may be an indicator of low cathepsin D expression.

Angiostatin is a potent antiangiogenic factor that is proteolytically derived from plasminogen (41). Angiostatin inhibits vasodilation, proliferation, and migration of endothelial cells and endothelial tube formation possibly via the induction of apoptosis in endothelial cells (41–45). Conditioned medium of human prostate carcinoma cells has been demonstrated to cleave plasminogen to angiostatin by the action of procathepsin D. Furthermore, purified mature cathepsin D can cleave plasminogen to angiostatin (46). We initially investigated the generation of angiostatin from plasminogen in conditioned media collected from COX-2 sense, COX-2 antisense, and wild-type cells. Angiostatin accumulation was abolished in the media from COX-2 sense cells and elevated in the media from the COX-2 antisense cells compared with the wild-type cells supporting a role for cathepsin D in the generation of angiostatin in endometrial epithelial cells. The differential cleavage of plasminogen to angiostatin in the three cell lines is reflective of the varying degrees of COX-2 expression. Coincubation of plasminogen in conditioned media from antisense cells with the cathepsin D inhibitor, pepstatin A, reduced angiostatin formation. This suggests that the cleavage of plasminogen to angiostatin is mediated in part by cathepsin D. Other proteolytic enzymes are known to cleave plasminogen to angiostatin. These include matrix metalloproteinases, plasminogen activators, pepsin, and cathepsin E (35, 46–50). Hence, it is plausible to suggest that, in addition to cathepsin D, other enzymes may be involved in the regulation of angiostatin production that may be inhibited by pepstatin A.

Overexpression of COX-2 and prostanoid receptors such as EP2 play a role in angiogenesis by promoting the formation of proangiogenic factors (51). Once synthesized the angiogenic factors act in a paracrine manner on endothelial cells to promote enhanced cell migration and tubular formation (12). However, it is now well accepted that the promotion of an angiogenic environment is the result of a balance in the production of angiogenic and anti angiogenic factors (52). Recently, inhibition of COX-2 has been demonstrated to upregulate the generation and expression of the antiangiogenic factors endostatin and thrombospondin-1 (53, 54). The data presented herein demonstrate an alternative pathway by which COX-2 can regulate angiogenesis in endometrial epithelial cells through inhibition of production of antiangiogenic factors such as angiostatin. Hence it is hypothesized that, in endometrial pathologies that are associated with elevated COX-2 enzyme expression, vascular function may be promoted through overexpression of angiogenic factors and reduced production of antiangiogenic factors such as angiostatin. However, the underlying cellular and molecular mechanisms by which COX-2 down-regulates cathepsin D expression resulting in reduced angiostatin generation remains to be elucidated.

In conclusion, the data outlined here demonstrate overexpression of COX-2 results in a concomitant induction of PGE_2 secretion and EP2/EP3 receptor expression. COX-2 inhibits cathepsin D expression in endometrial epithelial cells via an unknown mechanism, which contributes to an inhibition of the formation of angiostatin. These data outline a novel function of COX-2 in promoting a proangiogenic environment through suppression of production of angiostatin.

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